

# **Characterization of PIM kinases and cancer stem cell features in trophoblast cells**

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## LIST OF ABBREVIATION

Akt	Synonym of PKB (Protein kinase B)
BAD	Bcl-2-associated death promoter
Bp	Base pairs
BSA	Bovine serum albumin
BrdU	Bromodeoxyuridine (5-bromo-2'-deoxyuridine)
cDNA	Complementary deoxyribonucleic acid
CASP	Caspases, or cysteine-aspartic proteases or cysteine-dependent aspartate-directed proteases
CAM	Cell adhesion molecule
CC	Cell column
CD	Cluster differentiation
CDX2	Caudal type homeobox 2
CL	Corpus Luteum
CLC	Cardiotrophin-like cytokine
CLL	Chronic lymphocytic leukemia
CO <sub>2</sub>	Carbon dioxide
CSC	Cancer stem cell
CTB	Cytotrophoblast
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DNase	Deoxyribonuclease
dNTP	Deoxynucleoside triphosphate
dT	Deoxythymidine
DTT	Dithiothreitol
EBS	Embryonic stem cell
ECM	Extracellular matrix
Ectb	Endovascular cytotrophoblast
EDTA	Ethylendiaminetetraacetic acid
EGF	Epidermal-Growth-Factor
EIF-4E	Eukaryotic translation initiation factor 4E
Ep-CAM	Epithelial cell adhesion molecule
EVT	Extravillous trophoblast
FBS	Fetal bovine serum
FSH	Follicle stimulating hormone
FSR	Follicle stimulating hormone receptor
Fwd	Forward
G	Gravitational acceleration
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GDM	Gestational diabetes mellitus
G-CSF	Granulocyte colony-stimulating factor
GM-CSF	Granulocyte-macrophage Colony-stimulating factor
Gp130	Glycoprotein 130
Grb-2	Growth factor receptor-bound protein 2

HBEGF	Heparin-binding EGF-like growth factor
hES	Human embryonic stem cells
HCG	Human chorion gonadotropin
HGF	Hepatocyte growth factor
HIF	Hypoxia inducible factor
HIF- $\alpha$	Hypoxia Inducible factor-alpha
HOXA9	Homeobox protein Hox-A9
Hpl	Human placental lactogen
Hsp	Heat shock proteins
ICM	Inner cell mass
Ictb	Interstitial cytotrophoblast
IL	Interleukin
IL-11	Interleukin- 11
IL-6	Interleukin- 6
IUGR	Intra-uterine growth restriction
JAK/STAT	Janus kinase/Signal transducer and activator of transcription
JNK	Jun N-terminal kinase
LE	Luminal epithelium
LH	Luteinizing hormone
LIF	Leukemia inhibitory factor
LIFR	Leukemia inhibitory factor receptor
MCL1	Myeloid cell leukemia sequence 1
MMP-2	Matrix Metalloproteinase-2
MMP-9	Matrix Metalloproteinase-9
MMPs	Matrix Metalloproteinases
MDR1	Multi Drug Resistant gene 1
MTS	3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium
mTOR	Mammalian target of Rapamycin
MYC	Myelocytomatosis viral oncogene
NaCl	Sodium chloride
NANOG	Homeobox protein nanOg
NCBI	National Center for Biotechnology Information
OSM	Oncostatin M
p-BAD	Phospho Bcl-2-associated death promoter
PAA	Polyacrylamide
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
POU5F1	POU domain, class 5, homebox 1
PKC	Protein kinase C
p-MYC	Phospho-myelocytomatosis viral oncogene
PVDF	Polyvinyl



qRT-PCR	Quantitative real time polymerase chain reaction
Rev	Reverse
Src	Sarcoma
sFlt-3	Soluble fms-like tyrosine kinase-3
SH2	Src homology 2
SOCS	Suppressors of cytokines signaling
SOS	Son of sevenless
SOX2	SRY (sex determining region Y)-box 2
sPIGF	soluble placental growth factor
SSE4A	Stage-specific embryonic antigen 4
STAT3	Signal transducer and activator of transcription
STB	Syncytiotrophoblast
VEGF	vascular endothelial growth factor
SYGR Green I	N', N'-dimethyl-[4-[(E)-(3-methyl-1, 3-benothizol-2-ylidene) methyl]-1-Phenylquinolin-1-ium-2-yl]-N-propylpropane-1, 3-diamine
TAE	Tris-acetate-EDTA
Taq	Thermus aquaticus
TBS	Tris-buffered saline
TEMED	N, N, N', N'- Tetramethylethylen diamine
TGF- $\beta$	Transforming growth factor beta
Tr	Trophoectoderm
VEGFA	Vascular endothelial growth factor A
v/v	volume to volume ratio
w/v	weight to volume ratio

## SUMMARY

### Part I

The proviral insertion in murine (PIM) lymphoma proteins are a serine /threonine kinase family composed of three isoforms: PIM1, PIM2 and PIM3 which play a critical role in the control of the cell survival, proliferation, homing, migration, apoptosis inhibition, micro-environmental signaling and drug resistance. Overexpression of PIM kinases has been reported in several human tumors, mainly in hematological cancers. PIM kinases are cytokine dependent and mainly regulated by the Janus Kinase/Signal Transducer Activator of Transcription (JAK/STAT) signaling pathway. This is mainly activated by members of the Interleukin-6 (IL-6) family, but also others. Also Leukemia Inhibitory Factor (LIF) and Oncostatin M (OSM) belong to this family. Different trophoblast subtypes form the interface between mother and fetus and exert a crucial role in implantation and placentation necessary for successful pregnancy. Several pathologies are related with excessive trophoblast invasion, such as placenta percreta or choriocarcinoma, or with shallow invasion such as preeclampsia. The major aim of this part of the thesis was to further investigate the involvement of PIM kinases in regulation of trophoblast behavior. This may help to understand not only the physiology of embryo implantation and placentation, but also to identify potential disorders, which may lead to pregnancy complications.

In the human immortalized first trimester extravillous trophoblast cell line HTR8/SVneo and in the choriocarcinoma cell line JEG-3, we have analyzed the mRNA expression of PIM1, PIM2 and PIM3 by quantitative real time-PCR, and their protein expression by Western blotting and immunocytochemistry. We have further investigated the expression kinetics after stimulation with LIF. We have inhibited PIM kinases by using SGI-1776 at different doses for up to 3 days. Subsequently, we have studied the kinetics of cell viability by MTS assays and the proliferation by BrdU assays. Development of apoptosis was analyzed by Western blotting for BAD, BCL-XL, (cleaved) PARP and CASP3 as also the expression and phosphorylation of the potential PIM target c-MYC. Additionally, apoptosis and necrosis was tested by flow cytometry by annexin V and propidium iodide binding.

The major results are that all analyzed PIM kinases are expressed in both cell lines and further increased upon stimulation with LIF. Inhibition of PIM kinases significantly reduces viability and proliferation and induces apoptosis as assessed by increased cleaved PARP and annexin V/propidium iodide binding. Simultaneously, phosphorylation of c-MYC was reduced.

These results demonstrate the involvement of PIM kinases in LIF-induced regulation in two different trophoblastic cell lines which may indicate similar functions in primary cells.

## Part II

Trophoblast cells have the ability to differentiate into embryonic lineages, which behave similar to tumor cells. In previous studies of our group, it has been demonstrated that trophoblast cells are able to form spheroids. We hypothesized that HTR8/SVneo-derived spheroids may have properties similar to cancer stem-like cells (CSCs). As per literature, formation of spheroids promotes proliferation, migration and invasion in tumors (or also in trophoblast cell lines). HTR8/SVneo cells were used as a model for the analysis of tumor-like behavior of trophoblast cells. Our aim was to study and characterize the cancer stem cell-like properties in spheroids formed from HTR8/SVneo cells and the effect of cytokines such as LIF and OSM upon stimulation. We have observed that the HTR8/SVneo cells forming spheroids expressed the stem cell surface marker  $CD44^{+ve\ high}/CD34^{+ve\ low}/CD24^{+ve\ low}$ . Upon LIF stimulation these markers showed a decreased expression. The stemness-related transcription factors NANOG and CDX2 were constitutively expressed. Upon OSM treatment the expression level of NANOG is increased and that of CDX2 decreased. Hence, we conclude that HTR8/SVneo cells contain a fraction or side-population that expresses stemness related factors. Functions and capabilities of these cells remain to be investigated.

# ZUSAMMENFASSUNG

## Teil I

Die "Proviral Insertion in Murine (PIM) Lymphoma" Proteine bilden eine Serin/Threonin Kinase Familie bestehend aus drei Isoformen: PIM1, PIM2 und PIM3. Diese spielen eine entscheidende Rolle bei der Kontrolle der Zellviabilität, der Proliferation, des "Homing", der Migration, der Apoptose und anderer Vorgänge. Die Überexpression von PIM Kinasen wurde in verschiedenen humanen Tumoren beobachtet, vorwiegend in hämatologischen. PIM Kinasen sind Zytokin-abhängig und werden vorwiegend durch Janus Kinase/Signal Transducer Activator of Transcription (JAK/STAT) Signalwege aktiviert. Diese werden vor allem von Zytokinen der Interleukin-6 (IL-6) Familie aktiviert, aber nicht ausschließlich. Auch Leukemia Inhibitory Factor (LIF) und Oncostatin M (OSM) gehören zu dieser Familie. Verschiedene Trophoblast-Subtypen formen die Grenzfläche zwischen Mutter und Fetus und spielen eine bedeutende Rolle in der Implantation und Plazentation und sind somit notwendig für eine erfolgreiche Schwangerschaft. Mehrere Pathologien sind assoziiert mit exzessiver Trophoblast-Invasion, so wie Placenta percreta or Chorioncarcinom, oder mit schwacher Invasion so wie Präeclampsie. Das Ziel dieses Teils der Arbeit war die weitere Untersuchung der Beteiligung von PIM Kinasen an der Regulation des Verhaltens von trophoblastären Zellen. Die Ergebnisse können dazu beitragen, nicht nur die Physiologie der Embryo-Implantation and Placentation weiter zu verstehen, sondern auch potenzielle Störungen, die zu Schwangerschaftskomplikationen führen können. Wir haben die humane immortalisierte extravillöse Ersttrimester Trophoblast-Zelllinie HTR8/SVneo und die Chorioncarcinom-Zelllinie JEG-3 als Modelle benutzt. Die mRNA-Expression von PIM1, PIM2 und PIM3 wurde mittels quantitativer real time-PCR, und die Protein Expression mittels Western blotting und Immunozytochemie gemessen. Wir haben darüber hinaus die Expressionskinetik nach Stimulation mit LIF untersucht. Wir haben die PIM Kinasen mittels verschiedener Dosen SGI-1776 bis zu 3 Tagen inhibiert. Anschließend haben wir die Kinetik der Zellviabilität mittels MTS Assays und die Proliferation mittels BrdU Assays untersucht. Das Fortschreiten der Apoptose wurde mittels Western blotting für BAD, BCL-XL, (gespaltenes) PARP und CASP3 analysiert sowie die Expression und

Phosphorylierung des potenziellen PIM Targets c-MYC. Zusätzlich wurden Apoptose und Nekrose durchflusszytometrisch durch Annexin V- und Propidiumiodid-Bindung bestimmt.

Die wichtigsten Ergebnisse sind, dass alle analysierten PIM Kinasen in beiden Zelllinien exprimiert werden, was durch Stimulation mit LIF weiter gesteigert wurde. Die Inhibierung der PIM Kinasen reduzierte signifikant die Viabilität und Proliferation der Zellen und induzierte signifikant die Apoptose, besonders deutlich durch den Anstieg an gespaltenem PARP sowie durch gesteigerte Annexin V und Propidiumiodid-Bindung. Gleichzeitig war die Phosphorylierung von c-MYC reduziert.

Diese Ergebnisse demonstrieren die Beteiligung von PIM Kinasen an der LIF-induzierten Regulation in zwei verschiedenen trophoblastären Zelllinien, was auf ähnliche Funktionen in primären Zellen hindeutet.

## Teil II

In früheren Studien unserer Gruppe konnten wir zeigen, dass Trophoblastzellen in der Lage sind Spheroide zu formen. Wir haben erwartet, dass HTR8/SVneo-Zell-Spheroide ähnliche Eigenschaften wie Tumor-Stammzellen entwickeln. In der Literatur wurde berichtet, dass die Bildung von Sphroiden die Proliferation, Migration und Invasion in Tumoren (oder auch in Trophoblastzelllinien) steigert. HTR8/SVneo-Zellen wurden von uns als Modell genutzt zur Untersuchung von tumor-ähnlichem Verhalten von Trophoblastzellen. Unser Ziel war, Tumor-Stammzell-ähnliche Eigenschaften in aus HTR8/SVneo-Zellen geformten Sphroiden zu untersuchen und die Effekte der Zytokine LIF und OSM zu testen. Wir haben beobachtet, dass HTR8/SVneo-Zellen in Sphroiden die Stammzelloberflächenmarker  $CD44^{+ve\ high}/CD34^{+ve\ low}/CD24^{+ve\ low}$  exprimieren. Nach Stimulation mit LIF zeigten diese Marker eine verminderte Expression. Die Stammzell-assoziierten Transkriptionsfaktoren NANOG und CDX2 wurden konstitutiv exprimiert. Nach Behandlung mit LIF stieg die Expression von NANOG und verminderte sich die Expression von CDX2. Wir schlussfolgern deswegen, dass HTR8/SVneo-Zellen eine Fraktion oder Neben-Population aufweisen, die Stammzell-assoziierte Faktoren exprimiert. Die Funktionen und Fähigkeiten dieser Zellen sollten in späteren Studien untersucht werden.

## CHAPTER 1 INTRODUCTION

### ***1.1 Pregnancy-An enigma of new life***

Pregnancy is a beautiful event of creation of new life on earth, which is accompanied by its own enigma towards its success and failure. So far, researchers are doing all the possible to unravel this mystery.

It goes back in the times when a famous scholar in the field of reproduction biology named Medawar in 1953 put these words into the world of science to solve the need of understanding pregnancy and its complications Billington (2003).

Pregnancy has its own charm to pull anyone towards its riddle and create quest to unwind the story. In literature pregnancy is defined as the period taking place up to 38-40 weeks from the time of its start.

Pregnancy has been always viewed as a close cooperative interaction and regulation between a mother and her fetus. During implantation, fetal derived cells (trophoblast) invade the maternal endometrium and remodel the endometrial spiral arteries into low-resistance vessels that are unable to constrict (Haig 1993) .

Migratory interstitial cytotrophoblasts play a role in the preparation of the myometrium segments of the utero-placental arteries for the second wave of endovascular trophoblast migration that occurs in the second trimester of human pregnancy (Pijnenborg et al. 1983).

Columns of trophoblast cells advance to the base of the implantation site where they spread out to form a cytotrophoblastic cell. In addition, cytotrophoblasts advance into the lumen of the spiral arteries. They are responsible for remodeling these vessels to form wide, low-resistance conduits. In human and great apes, there is additional invasion of the endometrium and its vessels by trophoblasts originating from the base of the anchoring villi. Deep trophoblast invasion that extends remodeling of the spiral arteries to segments in the inner myometrium evolved in the common ancestor of gorilla, chimpanzee and human (Carter et al. 2015).

## ***1.2 Stages in Pregnancy***

During pregnancy, the woman undergoes many physiological changes including hematological, renal, metabolic, cardiovascular and respiratory. Human pregnancy is divided into three stages or trimesters. The first twelve weeks of pregnancy are termed to be first trimester which includes fertilization of egg travelling down the fallopian tube and attachment, invasion of the blastocyst into the endometrium of the uterus. There, the embryo and placenta start their journey with several modifications in terms of immunological tolerance and building a favorable environment for their development (Duc-Goiran et al. 1999).

There is a high risk of miscarriage during the first trimester. During first trimester, a large number of cells expressed by the decidua are leukocytes, among that the largest single population are natural killer cells with smaller numbers of macrophages and T cells (Hohn und Denker 2002). However by the end of pregnancy these NK cells are the smallest cell population left (Starkey et al. 1988). The second trimester is from week thirteen to twenty eight, where the movement of fetus can be felt by the mother. The third trimester is from week 29 to 40.

## ***1.3 Ovulation and Implantation***

After the woman's last day of the menstrual period cycle, the uterus begins a proliferative phase until the rise of the follicle stimulation hormone (FSH) that subsequently leads to ovulation in order to give rise to mature oocyte. The fusion of this egg cell with a male gamete to form a zygote is called fertilization. This stage is also considered an event of initiation of the pregnancy.

In humans implantation has been conceptualized as a stepwise process involving apposition and adherence of a blastocyst to the endometrium, followed by breaching of luminal epithelium and finally invasion into maternal tissues.

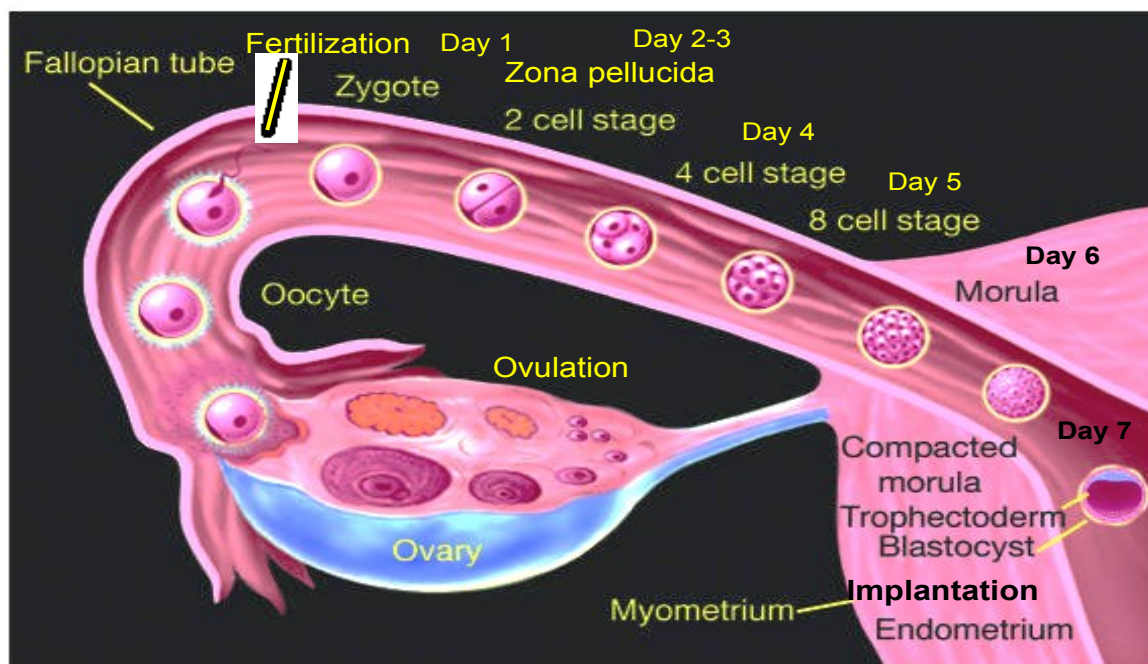
In order for implantation to happen, endometrium has to undergo changes and becomes decidua (Salamonsen et al. 2003), which is accomplished by the modification of endometrial stromal cells, uterine vessels and glands as well as immune cells (Lunghi et al. 2007). From (Figure.1) it is shown after fertilization, the embryo develops from morula to blastocyst (after day 5), a spherical structure composed of cells called inner cell mass (ICM), and outside of it is trophoblast cells. The ICM subsequently develops into the fetus.

Implantation starts after the hatching of the blastocyst to the zona pellucida (day 6). window of implantation (WOI), is characterized by two situations: 1. Endometrium receptivity (Duc-Goiran et al. 1999), 2. Blastocyst invasion competence as a result it breaches the uterine epithelium (day 7). The first cell-to-cell contact can be recognized when apical plasma membranes of trophoblast and uterine epithelial cells adhere (Hohn und Denker 2002) (Poehlmann et al. 2005a). The expression of adhesion molecules such as integrin's, adherins, selectin and immunoglobulin superfamily, is involved in this process.

The ICM begins to differentiate into different layers and begins to produce human chorionic gonadotropin (HCG), a hormone that insures the implanting embryo. The trophoblast mediates the attachment and invasion into the uterine wall over next few weeks. Thereby, placenta develops and continues production of progesterone initiated by corpus luteum that controls and maintains decidualization. The decidua is the uterine basis of the mother guaranteeing that the fetus is provided with nutrient and oxygen needed for its successful growth and development. (Norwitz 2006) (Damjanov 2014). Progesteron has been implicated in immunosuppression at the feto-maternal interface, hence protecting the fetus from unwanted immunological response. Due to higher secretion of progesterone from the corpus luteum (CL), the secretory phase replaces proliferative phase in order to prepare the endometrium for implantation by stimulating endometrial glands (Lee et al. 2011a) (Szekeres-Bartho et al. 2009) (Santoro et al. 2000). Implantation is regulated by a complex interplay between endometrium and trophoblast.



The endometrium controls the trophoblast invasion by secreting cytokines and protease inhibitors, which in turn modulate the invasion of trophoblast. Less HCG is produced due to the closer presence of trophoblast to the endometrium, allowing further differentiation of trophoblast into anchoring type cells. Decidualization continues and regulates the placental formation and the expression of regulatory factors such as surface antigens, metalloproteases and major histocompatibility (MHC) molecules (Hess et al. 2007). Decidua basalis is characterized by infiltration of immune-competent subsets cells like natural killer cells (NK) cells, progenitor cells, some T cells (Trundley und Moffett 2004), macrophages and a few B cells. Decidua in simple terms may be capable of inducing an immunological reaction against the implanting blastocyst, or later placenta. These cells produce a respective cytokine profile regulating implantation and trophoblast invasion (Saito 2000).

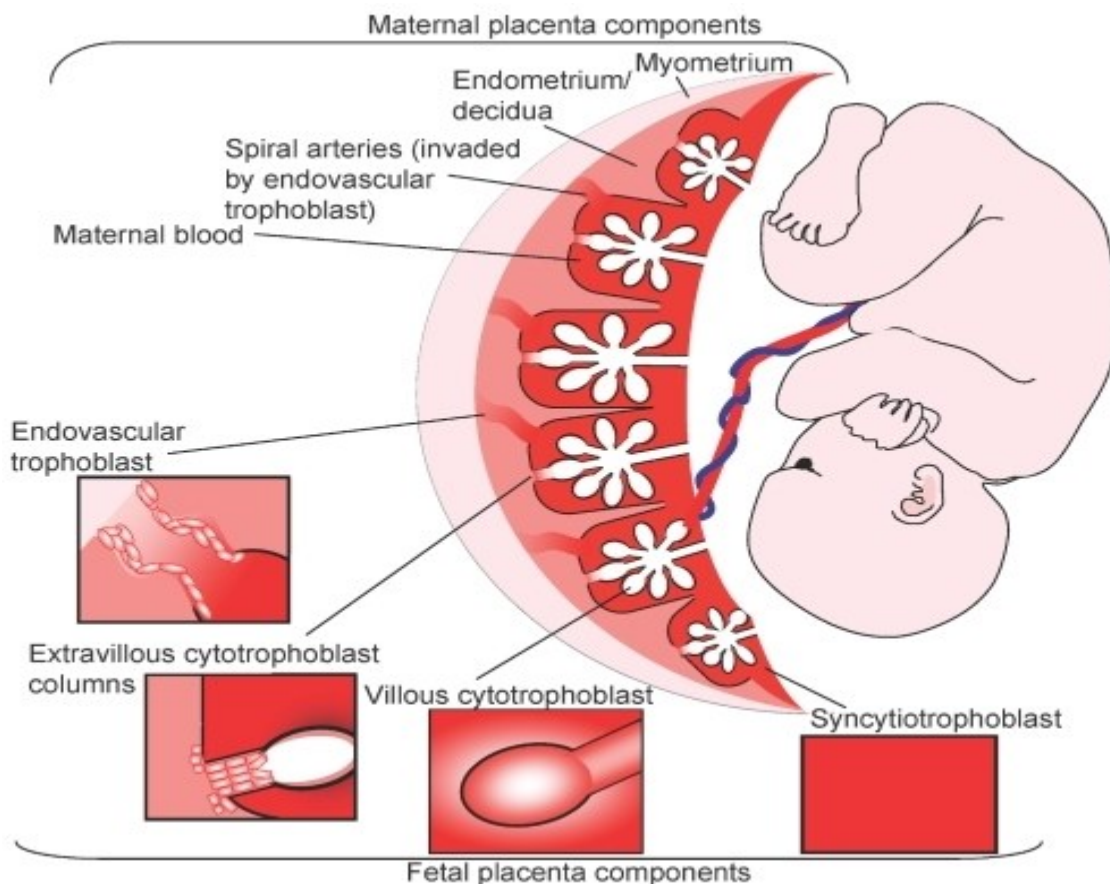


**Figure 1.** Ovulation and implantation.

Fertilization occurs in the fallopian tube within 24 to 48 h of ovulation. The initial stages of development, from fertilized ovum (zygote) to a solid mass of cells (morula), occur as the embryo passes through the fallopian tube encased within a non-adhesive protective shell (the zona pellucida). Modified (Red-Horse et al. 2004)

## 1.4 Placentation

Placenta is an organ that is transient and depicted as a simple disc-shaped structure which is rapidly assembled in the extra-embryonic compartment (Hunkapiller und Fisher 2008). Humans have a hemochorial placenta, where blood from mother comes in direct contact with fetal derived trophoblast cells. Chorionic villi are sprout in order to give a maximum possible area of contact within the maternal blood, thus these villi are the interface between mother and fetus. Placenta cells produce several peptide and steroid hormones (Tuckey 2005), cytokines, growth factors and other bioactive factors. Placental functions can be viewed as different aspects of protection such as against dehydration, toxic metabolites, malnutrition (Evain-Brion und Malassine 2003) (Myllynen et al. 2005).

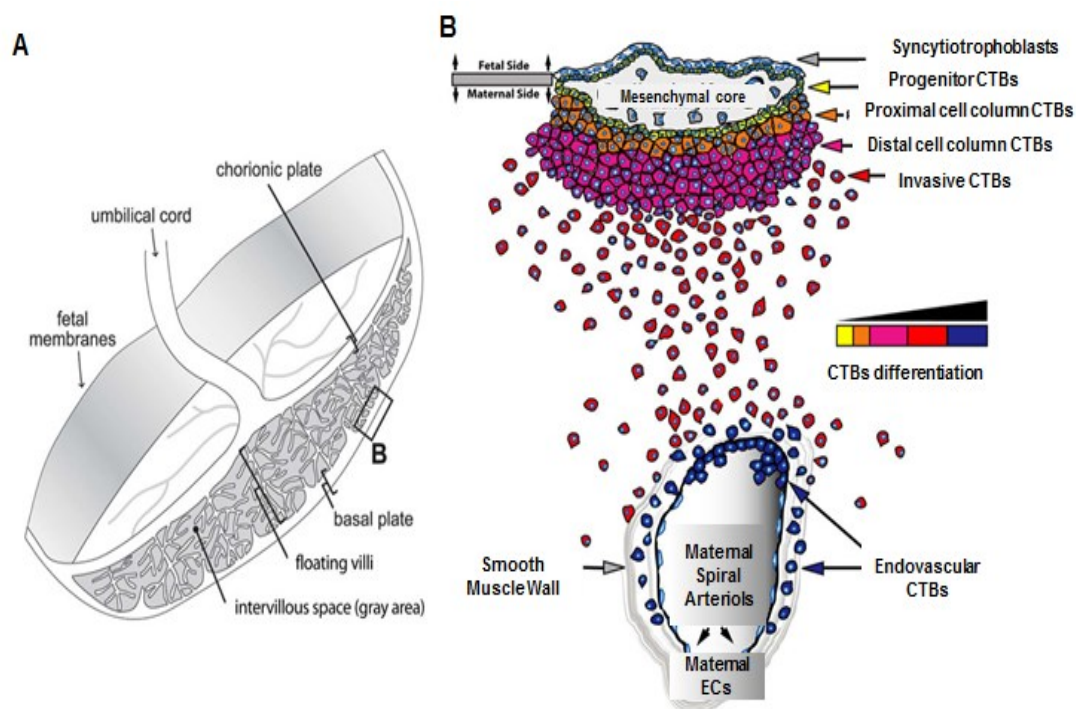


**Figure 2.** The human placenta.

Villous trophoblasts of the human placenta grow as a branched structure, maximizing exchange with maternal blood. Extra villous trophoblast invades into the maternal endometrium Modified (Frost und Moore 2010).

Trophoblast cells are embryonic tissue formed during early stage of pregnancy deriving from the “trophoectoderm” (Figure.2). They form the outer layer of the blastocyst, providing the nutrients to the embryo and further develop into large part of placenta (Haig 1993). Trophoblast further proliferates and differentiates into two layers. 1). Cytotrophoblast (CTB): Single celled, inner layer of the trophoblast (Bischof und Irminger-Finger 2005), which further develops into extra-villous trophoblast cells (EVT) formed from the tip of the villi, growing out from placenta, penetrating the decidualized uterus (Handwerger 2010) and 2). Syncytiotrophoblast (STB) a multinucleated cell complex (Mi et al. 2000) (Fisher et al. 1989) that divides maternal and fetal blood streams in gestation, also secreting human chorionic gonadotropin (HCG) (Soncin et al. 2015) (Faas et al. 2014). STB is formed by the fusion of cytotrophoblasts and is responsible for initiation of invasion of endometrium (Faas et al. 2014). (Fig 3) The deeper penetration of maternal tissues is achieved by cytotrophoblasts. Both cells resemble to each other and can be identified histochemically (Haig 1993). One of the modulating mechanisms during villous branching is by the human placental macrophage (Hofbauer cell) (Anteby et al. 2005). The arterioles that supply blood to the endometrium becomes highly coiled are known as spiral arteries (Fig 4). They transport the maternal blood to the placenta. The maternal tissues that line the uterus are called decidua. They shed with the placenta during delivery (Haig 1993). The hemochorial placentation (Fig 5 ) which requires an epithelial-to-endothelial conversion (Iruela-Arispe 1997) and it arises primarily through differentiation, proliferation, migration and invasion of the endometrium and its vasculature by the CTB (Duc-Goiran et al. 1999). Similarities have been frequently asserted between growing fetus and invading cancer (Quenby und Brosens 2013) (Pollheimer und Knofler 2005) (Koldovsky 1999) in terms of invasion, migration and proliferation. Malign cells have similarities with CTB and EVT, which show highly invasive characteristics especially during implantation and first trimester of pregnancy: There is involvement of autocrine/paracrine communications, adhesion molecules and protease profiles. In healthy pregnancy the invasive growth by EVT is restricted to the uterus of the first trimester (Lunghi et al. 2007). EVT cells enter the decidua and subsequently later the myometrial stroma as interstitial trophoblast (Brosens et al. 2002).

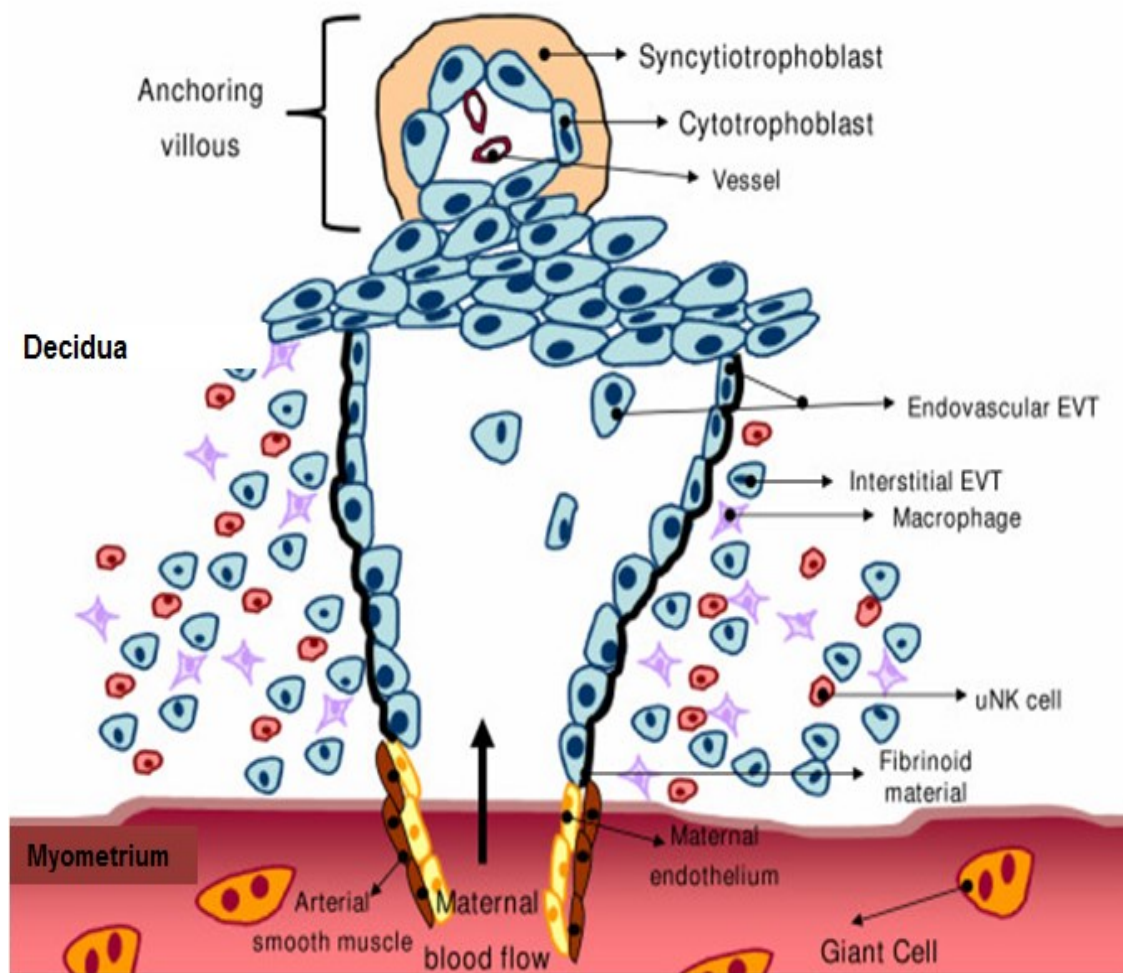
There they form giant cells and lose invasiveness. EVT also surround and destroy smooth muscle cells (SMC) of spiral arteries hence replacing the fibroid material. Further, EVT invade the lumen of the arteries to replace the endothelium of the vessels (Zhou et al. 1997). MHC class I molecules are expressed in EVT cells, in particular human leukocyte antigen HLA-C, E and G which are good ligands for many killer immunoglobulin receptors (KIR) present on NK cells. Such interactions modify the cytokine receptors and regulate adhesion molecules as well as Matrix Metallo Proteinases (MMP) (Matthiesen et al. 2005). MMPs,  $\alpha 5 \beta 1$  integrin's, VE-cadherin's and trophoblast specific HLA class I (e.g. HLA-G) support EVT invasion. Although the invasiveness of the trophoblast is known to be regulated in local and temporal terms, the regulation of these activities is also related to differentiation pathways leading to formation of non-invasive villous trophoblast serving endocrine as well as nutritive functions (Hohn und Denker 2002).



**Figure 3.** Placental chronic villi

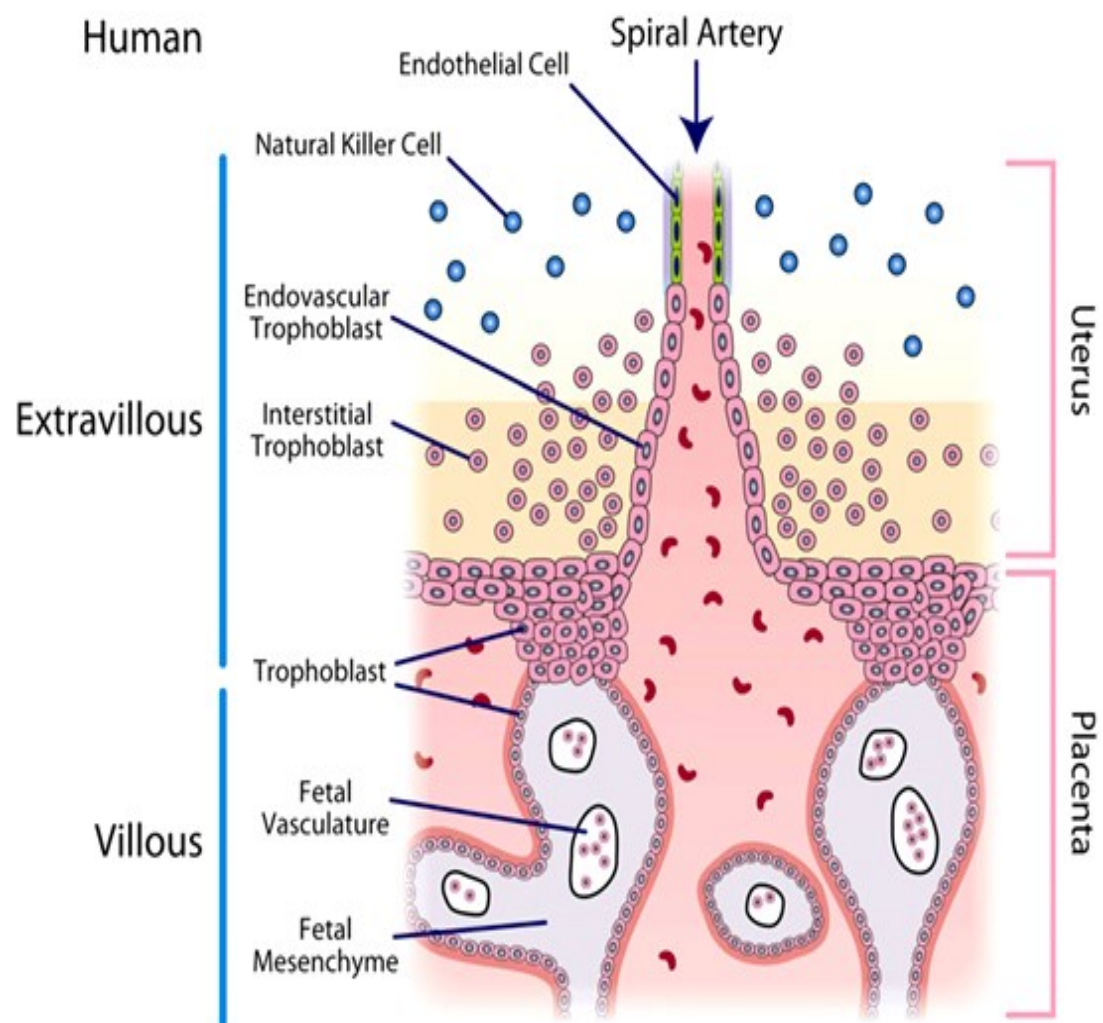
Stem from the chorionic plate and lie within the intervillous space. The point of attachment between anchoring villi and the underlying tissue is the basal plate (box B). Enlargement of the area in box. Undifferentiated CTB in the anchoring villi give rise to invasive CTBs that invade the uterine interstitium (interstitial invasion) and the maternal endothelium (endovascular invasion). Modified (Hunkapiller und Fisher 2008)





**Figure 4.** Spiral artery remodeling

Maternal spiral artery remodeling through the combined action of interstitial and endovascular extravillous trophoblast cells. EVT cells invade uterine wall and maternal spiral arteries replacing smooth muscle with fibroid material and part of vessel endothelium, thus evoking artery dilatation. Decidual immune cells, like macrophages and NK cells facilitate deep invasion of EVT cells up to myometrial portions of spiral arteries. Modified (Lunghi et al. 2007).



**Figure 5.** Hemochorial Placentation

Schematic diagram showing structures within human hemochorial placentation sites.  
Modified (Soares et al. 2012)

### ***1.5 Soluble factors involved in pregnancy: hormones, growth factors, cytokines***

A large number of hormones, growth factors and cytokines (Figure 6) are produced by both the pre-implantation embryo and the maternal endometrium: hormones like HCG, progesterone, growth factors like TGF, cytokines and receptors (Duc-Goiran et al. 1999). Pregnancy hormones are important for maintenance of the corpus luteus acting on luteotropic hormone that stimulates the production of progesterone via luteinizing hormone-receptors (LHR). Estrogen regulates uNK cells in the decidualization process, which helps the fetus to attach to the endometrium (Gibson et al. 2015). Human placental growth hormone (hPGH) and hPlacental lactogen (hPL) are released into both maternal and fetal circulation. They are found in the syncytiotrophoblast layer of placenta helping in stimulating the insulin-like growth factor (IGF) production and modulating metabolism, resulting in increased availability of glucose and amino acid to the fetus (Handwerger und Freemark 2000).

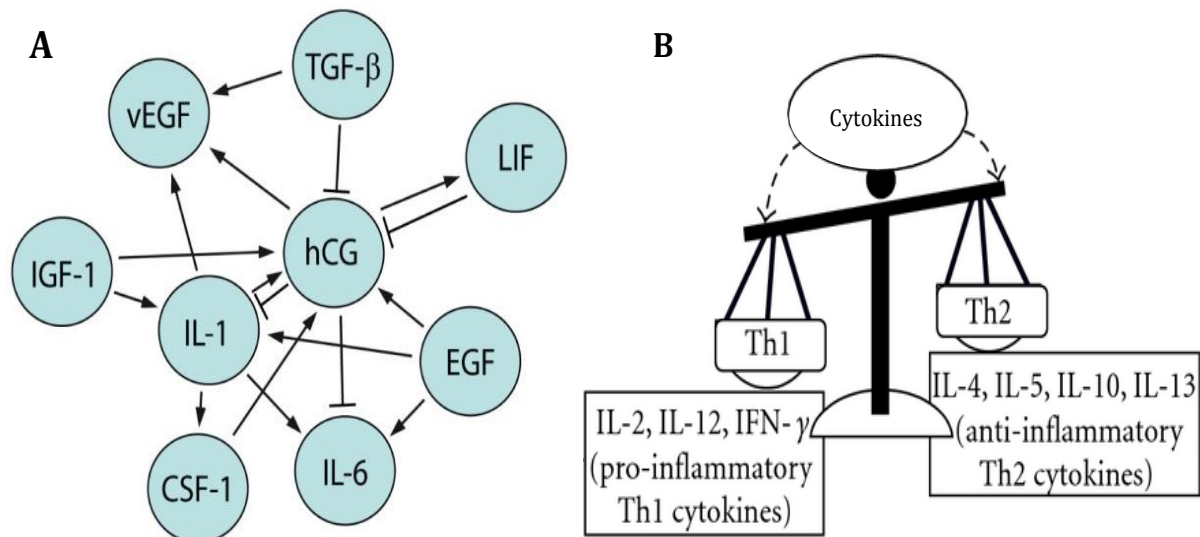
Growth factor expression/secretion is stimulated by paracrine interactions of diverse maternal and placental cell types. Predominant growth factors of the fetal-maternal interface as well as their mutual stimulations are shown in below Figure 6. During the early stage of blastocyst, epidermal growth factor (EGF) and transforming growth factor (TGF) are co-expressed with EGF-receptors (EGF-R) suggesting an autocrine function in human development. Platelet-derived growth factor (PDGF) with its two receptor subunits,  $\alpha$  and  $\beta$  is expressed from 8-cell stage onwards, while blastocyst secretes IGF factor II (IGF-II) (Srivastava et al. 2013). With the exception of TGF $\beta$  all factors depicted were shown to positively influence trophoblast proliferation and/or migration/invasion. Some soluble ligands such as HCG may play key roles in trophoblast motility since several growth factors trigger their secretion and also promote trophoblast migration through elevation of VEGF and LIF secretion. Hence, when studying effects of a particular growth factor on trophoblast migration direct as well as indirect effects must be considered. Colony stimulating factor I (CSF-I), similar to EGF and TGF, promotes EVT proliferation and stimulates production of MMPs and TIMPs in EVT. VEGF is synthesized by decidua macrophages and promotes EVT cell proliferation.

Cytokines are small multifunctional proteins often derived from leukocytes and have primarily been described through their immunomodulatory actions. The maternal–fetal interface is considered to be immunosuppressed to allow development of the semi-allogeneic placental fetal unit. However, cytokine profiles of the decidua and different decidual cell types suggest that the situation *in vivo* might be more complex than *in vitro*. Cytokines do not only play a role in immunoregulation, but also in other aspects of the establishment of pregnancy, including the regulation of trophoblast invasion and spiral artery remodeling. Dysregulation of decidua-derived cytokines may be involved in the etiology of unexplained spontaneous miscarriage (Lash und Ernerudh 2015). Cytokines are regulated at the transcriptional and translational level, usually leading to an altered proliferation, and differentiation in the target cell. (Table 1) One of the main functions of cytokines is regulation of inflammation, typically mediated by Th1 type (pro inflammatory) and Th2 type (anti-inflammatory). Representative Th1 type cytokines are interleukin 2 (IL-2) or interferon (IFN)  $\gamma$ . Th2 type cytokines are IL-4, IL-10, IL-13 and others.

**Table 1.** Summary of some assorted cytokine profiles during pregnancy

Name	Function
<b>TNF- <math>\alpha</math> (Toder et al. 2003)</b>	Activates intercellular signaling in embryonic development
<b>IFN- <math>\gamma</math> (Resch et al. 2004)</b>	Release of prostaglandins
<b>IL-1 (Prutsch et al. 2012)</b>	Interacts with endometrial glandular epithelial cells, trophoblast cell motility
<b>IL-6 (Prins et al. 2012)</b>	Expressed in endometrial stromal cells and involved in pro-inflammatory reactions, supports trophoblast invasion
<b>IL-8 (Jovanovic et al. 2010)</b>	Trophoblast cell migration and invasion





**Figure 6.** Cytokines, Hormones and Growth factors.

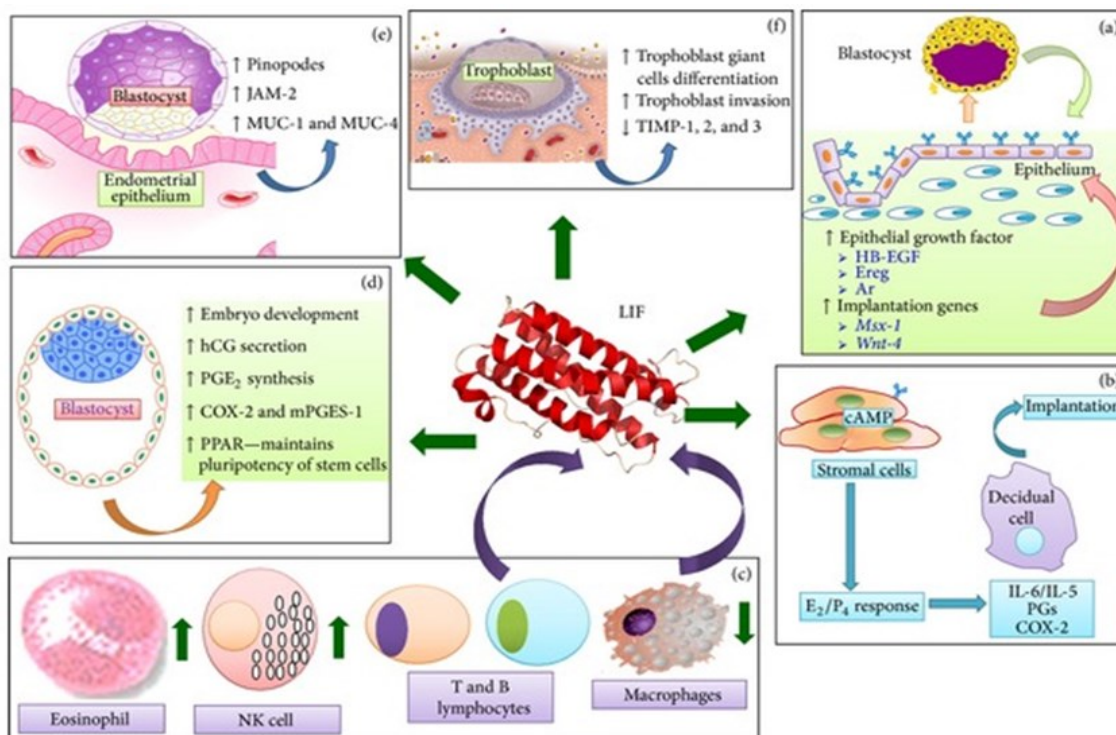
Interplay between growth factors expressed at the fetal-maternal interface. Stimulating (arrows) as well as inhibitory effects on expression / secretion are depicted. Modified (Knofler 2010). B. Cytokines in embryo growth and development (Yessoufou und Moutairou 2011).

## 1.6 LIF in pregnancy

Leukemia Inhibitory factor (LIF), is a secreted multifunctional glycoprotein belonging to the IL-6 super family, regulating various cellular function via binding to the membrane bound ligand receptor of LIF-receptor (LIFR) and gp130 (Mathieu et al. 2012). Its production is unregulated by progesterone and IL-4, and down regulated by various inflammatory mediators like IFN- $\gamma$ . Successful embryo implantation depends on an ideal cross talk between embryo and receptive endometrium which includes bilateral secretion and reception of LIF. It is produced by endometrium and blastocyst regulates growth and development of the embryo, There are three splice variants of LIF, which include diffusible, membrane-associated and truncated forms acting in paracrine fashion (Aghajanova 2004). Throughout the period of implantation, ovarian steroids regulate the expression of LIF, LIFR and gp130 *in utero* (Sherwin et al. 2004). In human, TGF- $\beta$  and HCG increase LIF secretion by endometrial epithelial cells form follicular and secretory phase during menstrual cycle

(Perrier d'Hauterive et al. 2004). LIF stimulates stromal decidualization by increasing the production of cytokines and prostaglandins.

LIF is also involved in enhancing embryo-endometrial interaction through pinopodes and adhesion molecules by stimulating trophoblast cell differentiation and increasing trophoblast capability to invade the uterine stroma. LIF acts as a pro-inflammatory cytokine and its decrease is involved in recurrent miscarriages' (Bischof und Irminger-Finger 2005) (Bischof und Irminger-Finger 2005). Noteworthy, LIF plays vital role in recruitment of a specific cohort of leucocytes which participates in inflammatory response during implantation. There are numerous factors (Figure 7) which are influenced due to the presence and absence of LIF during embryo and endometrial interaction, such as: heparin binding-epidermal growth factor (HB-EGF), epiregulin, amphiregulin, estrogen, progesterone, interleukins (e.g. IL-5, IL-6), prostaglandins (e.g. E2), cyclooxygenase (Lee et al. 2011b), NK cells, peroxisome proliferator-activated receptor, HCG or mucin (which is a glycocalyx expressed at the apical membrane of luminal epithelial and its expression is reduced during trophoblast invasion) (Sharma und Kumar 2012), junctional adhesion molecules (Su et al. 2012), Matrix metalloproteins (MMPs) and Tissue inhibitory proteins (TIMPs) (Wang et al. 2002a);(Song et al. 2000);(Salleh und Giribabu 2014) as shown in (Figure 7).



**Figure 7.** Summary of the known roles of LIF in embryo implantation.

LIF increases the expression of implantation genes in receptive endometrium. Modified (Salleh und Giribabu 2014).

### 1.7 Signaling network-mechanism

During human pregnancy, implantation and invasion of placental trophoblast into uterine wall is an essential event which is remarkable, flexibility of the trophoblast in fulfilling various functions such as secretions of hormones, anchorage of the placenta, modulating the decidual angiogenesis/ morphogenesis and spiral artery remodeling of maternal tissues, which requires increase blood flow (Knofler 2010). These processes are mediated by a complex network of signaling and adhesive factors (Massuto et al. 2010). Several extracellular stimuli (Table 2) initiate intracellular signal transduction upon interaction with receptor tyrosine kinase (RTKs), G-coupled receptors (GPCRs) and others, which ultimately leads to the activation of critical signaling cascades.

**Table 2.** Summary of signaling pathways in human

(Soncin et al. 2015); (Knofler 2010); (Gundogan et al. 2011); (Busch et al. 2009).

Signaling factor	Cell/Tissue Type	Function
<b>FAK</b>	First trimester CTB	Increased differentiation into EVT
<b>HGF/c</b>	trophoblast	Promotes EVT migration
<b>Notch</b>	First trimester CTB and explants	Variable effects (ECM-dependent)
<b>PKA</b>	Term CTB	Promotes STB differentiation
<b>PI3K/Akt</b>	Third trimester cell line	Promotes EVT migration
<b>PPAR<math>\gamma</math></b>	First trimester and term CTB	Inhibit invasion of EVT, promotes STB differentiation
<b>Wnt</b>	Choriocarcinoma cell line, first trimester CTB	Promote cell-cell fusion and stimulates migration/invasion of EVT
<b>Activin/Nodal</b>	First trimester	Inhibits cell invasion, proliferation and differentiation of EVT anchoring, induce apoptosis
<b>mTOR</b>	Immortalized first trimester trophoblast cell	Mediates trophoblast invasion through MMP

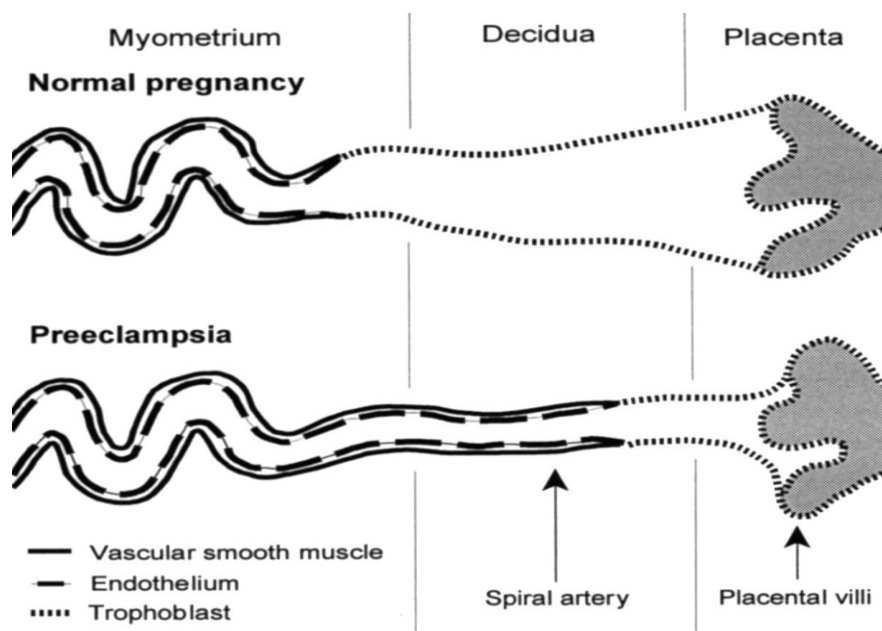
To understand the molecular networks involved in regulating an immune response, the cytokine receptor signaling and transduction pathways have been extensively studied and reported. Cytokines are regulators of embryogenesis and participate in inflammation triggering the onset of labor in late pregnancy (Orsi 2008). The Janus kinase/signal transducer activator of transcription (JAK/STAT) signaling network provides a very fast intracellular signal from a receptor to the nucleus. JAK/STAT consist of three main components: 1. Receptors, 2. Janus kinases, 3. STATs. The receptors are triggered by growth factors, interferon, interleukins and other chemical messengers which thereby, further activates JAK upon auto phosphorylation (SH2 domain binding) which then activates the STATs (SH2-domain possessing protein binds to the phosphorylated receptor, in turn STAT becomes phosphorylated (Aaronson und Horvath 2002). STATs are described as ligand-induced transcription factors (Darnell et al. 1994);(Darnell 1997);(Murray 2007).

Encoding genes and alternative post translational proteolytic cleavage generate additional forms (Jatiani et al. 2010) of STAT1, STAT2, STAT3 (Schaefer et al. 1995), STAT4 (STAT-4 $\alpha$  and STAT-4 $\beta$ ) and STAT5 (STAT-5a and STAT-5b) (Lin et al. 1996). EVT cells resemble tumor in their invasive and destructive properties, mainly during first trimester of pregnancy. LIF provides an extracellular signal stimulating invasion in trophoblast cells and induces STAT3 DNA binding activity in choriocarcinoma cells. STAT3 phosphorylation correlates with trophoblast invasiveness (Corvinus et al. 2003); (Poehlmann et al. 2005a). IL-11 activates STAT3 in choriocarcinoma cells and increases invasion (Suman et al. 2009). STAT3 activation induces expression of suppressors of cytokine signaling (SOCS3). Its absence is lethal to the embryo due development of placental insufficiency, hence SOCS3 seems to be important for pregnancy by regulating LIF-driven trophoblast differentiation (Fitzgerald et al. 2009). STAT3 activation is sustained by IL-10 expression in trophoblast cells resulting in increased cell motility (Dallagi et al. 2015).

### ***1.8 Pathologies in pregnancy***

To achieve successful pregnancy, it requires well-coordinated implantation of the embryo into the receptive decidua, placentation, trophoblast invasion of the maternal decidua and myometrium in addition to remodeling of the uterine spiral arteries. Failure of any of these steps can lead to a range of pregnancy complications, including miscarriage, infertility, pre-eclampsia, inter-uterine growth restriction (IUGR), placenta accrete, placenta previa, pre-term birth (PTB), pre-term labor (PTL) (Lash und Ernerudh 2015) or choriocarcinoma. Recurrent pregnancy loss (RPL) (Shahine und Lathi 2015); (Gammill et al. 2015) is a multifactorial condition of 3 or more consecutive pregnancy losses that affects 1-5% couples. Infertility is a very common complication defined as the inability to establish successful pregnancy within a certain period of time. Preterm birth is defined as before 37<sup>th</sup> week of pregnancy, which in worse cases may cause neonatal death or long term handicaps infants (Simons und Schatz 2012), It may be induced by insufficient trophoblast invasion (Hoesli et al. 2002). IUGR is a complicated disorder with varying etiology in pregnancy, characterized by failure of the embryo to accomplish its normal growth, hence resulting in morbidity and cardiovascular disease in adult life, it is mainly associated with placental dysfunction (Gourvas et al. 2012).

Preeclampsia is defined as high blood pressure, proteinuria and inflammation with an onset in the second half of pregnancy, it may occur as a result of abnormal placentation (Figure 8) due to loss of trophoblast differentiation. It has a high incidence in developed countries (Scioscia et al. 2015); (Sifakis et al. 2015). Choriocarcinoma is a rare but an aggressive and destructive malignant form of trophoblastic neoplasm that is associated with distant metastasis (Emin et al. 2015); (Froeling und Seckl 2014), it arises from hydatidiform mole, hyperplasia of trophoblast and villous cistern formation (Feist et al. 2015), although choriocarcinoma is not common in Europe or US but its prevalence is found ten times higher in Asian, African and south American countries. All gestational trophoblastic diseases (GTD) produce HCG, which often serves as diagnostic tool being a good parameter for progress of the disease or success of therapy (Khoo 2003).



**Figure 8.** Pathology during pregnancy

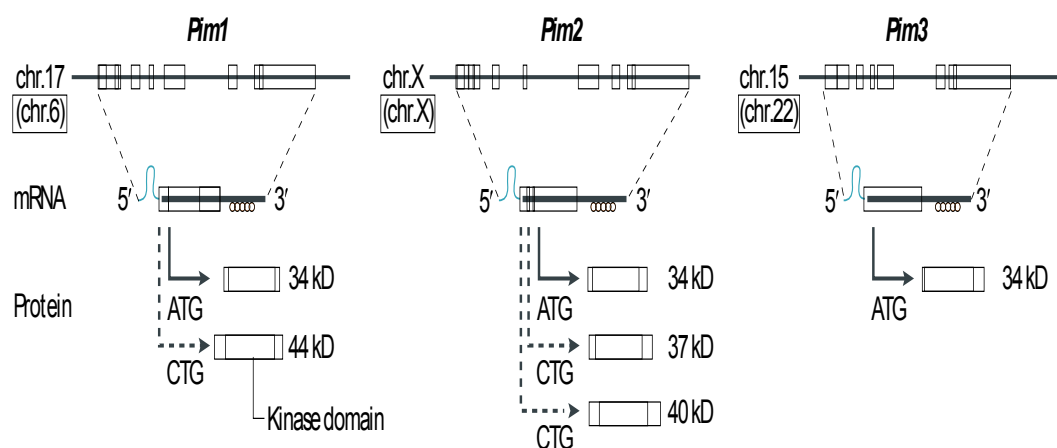
Trophoblast invasion into the spiral arteries in the placental bed in normal pregnancy and in preeclampsia, modified (VanWijk et al. 2000).

## 1.9 PIM kinases

Proviral insertion sites in moloney murine leukemia virus or *PIM* genes were identified as oncogenes in mid 1980s nearly three decades ago. PIM proteins belonging to serine/threonine kinases family consist of three different isoforms (PIM1, PIM2 and PIM3) and are highly evolutionary conserved (Narlik-Grassow et al. 2014); (Nawijn et al. 2011).

## 1.10 Structure and Function

The three isoforms of PIM kinases (PIM1, PIM2, PIM3) (Figure 9) (Nawijn et al. 2011). PIM genes are located at different chromosomal locations in humans. PIM mRNA has short half-life span owing to the presence of multiple copies of the destabilizing 3' untranslated region (UTR) (Feldman et al. 1998). Human *PIM1* and *PIM2* have alternative translational sites, resulting in the presence of two (*PIM1*) and three (*PIM2*) protein isoforms; the subcellular localization and substrate interactions are isoform specific. PIM1 and PIM3 share high degree of homology and 71% are identical at the amino acid level, PIM1 and PIM2 are 61% identical (Nawijn et al. 2011). PIM1 kinase encodes 33 and 44 kDa isoforms that have comparable kinase activity (Saris et al. 1991).



**Figure 9.** Structure of PIM Kinases

PIM1, PIM2 and PIM3, modified (Nawijn et al. 2011)

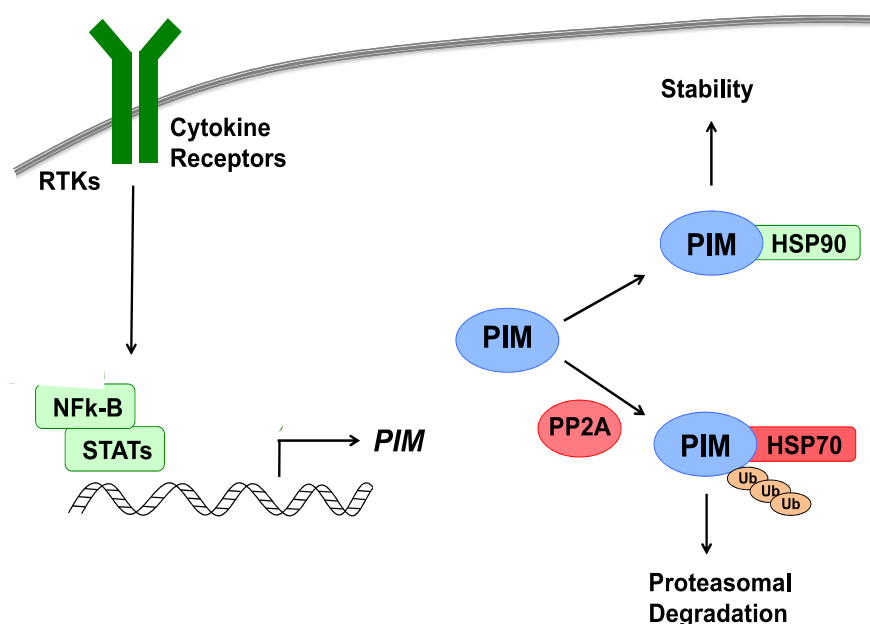
The larger isoform localizes to the plasma membrane whereas the shorter isoform is primarily localized in the nucleus (Xie et al. 2006). PIM1 is highly expressed in hematopoietic, gastric, head and neck tumors (Bachmann et al. 2006). PIM2 has three isoforms (34, 37, and 40 kDa), with no protein functions or interaction reported so far and is highly expressed in lymphoid and brain tissues (Cohen et al. 2004). PIM3 kinase has only one isoform (Nawijn et al. 2011) and is highly expressed in kidney, breast and brain tissues.

### ***1.11 Signaling pathways mechanism***

PIM kinases are mainly responsible for cell cycle regulation, anti-apoptotic activity and the homing and migration of receptor tyrosine kinases mediated via the cytokine dependent JAK/STAT pathway (Blanco-Aparicio und Carnero 2013) and NFkB (Zhu et al. 2002). PIM kinase gene expression is described in many cell types such as in neuronal, hematopoietic, endothelial, vascular smooth muscle, epithelial cell lineages (Gapter et al. 2006), cardiomyocytes, as well as in early progenitors cells (Cottage et al. 2010) and embryonic stem cells (Fischer et al. 2011); (Cottage et al. 2012). The PIM kinase gene expression is largely regulated at the transcriptional and translational level. PIM kinases act downstream of multiple oncogenic tyrosine receptors, Janus kinase (JAK) (Wernig et al. 2008) and FMS-like tyrosine 3 (FLT3) (Kim et al. 2005). JAK/STAT pathways play a critical role in regulation of the expression of PIM genes and represent alternatives to the secondary messenger signaling. When cytokines bind to a cell membrane receptor, JAKs phosphorylate the receptor domain in the cytoplasm, creating an intracellular binding site for STATs which in turn bind to phosphorylated JAK, leading to STAT dimer formation and translocation into the nucleus, where STATs induce transcription. STAT3 specifically binds to promoter sequences of PIM1, enhancing its transcription and expression in response to a variety of cell mitogens such as interleukins and growth factors. Interestingly, PIM activates SOCS proteins and functions as part of negative feedback mechanism that serves to restraint JAK/STAT network (Peltola et al. 2004). At the translational level the expression of PIM kinases is mainly regulated by the stability of mRNA. PIM mRNA transcripts have very short half-life as mentioned earlier and due to which the AUUU (Haig 1993) sequence located in the 3' UTR is destabilized.



The regulation of protein stability is critical for the cellular function and activity. PIM kinases have a short half-life (less than 5min), (Figure 10) their stability is mainly controlled through ubiquitination and proteosomal degradation while binding to heat shock protein (HSP70 and HSP 90) regulates PIM stability (Mizuno et al. 2001) ; (Shay et al. 2005). The initiation and progression of human cancer is linked to uncontrolled activation of survival kinases and two such pro-survival kinases that are commonly amplified in cancer are Akt (Protein kinase B /PKB) and PIM. So we are going to illustrate about PIM regulation with respect to cancer. PIM are oncogenic and have multiple functions (Narlik-Grassow et al. 2012). Altogether, these above mentioned studies show that PIM kinases may be constitutively active in cells and their activity can be influenced by phosphorylation by themselves or by other kinases. SOCS1 stability is regulated by PIM kinases by inducing JAK/STAT pathways and transcription of PIM kinases as induced by T cell antigen cross linking and cytokines such as IL-4, IL-6 and IFN- $\gamma$  (Chen et al. 2002).



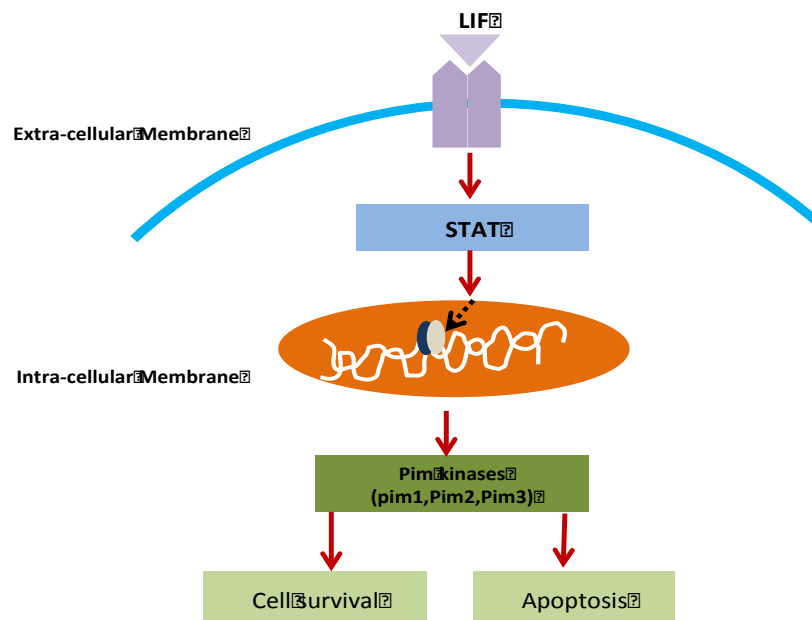
**Figure 10.** Regulation of PIM Kinases

mRNA and protein level in the presence of cytokines modified (Warfel und Kraft 2015).

### **1.12 PIM kinases in Cancer**

PIM kinases play important roles in regulating tumor cell proliferation by influencing cell cycle progression via p21 (Zhang et al. 2007), p27 (Wang et al. 2002b), p53 (Hogan et al. 2008) and CDC25C (Bachmann et al. 2006). One of the primary machineries of survival and apoptosis for PIM kinases is elicited through their pro-survival effect via the Bcl-2 family members, that has both pro-apoptotic (BAD and BAX) and anti-apoptotic (BCL-2 and BCL-XL) effects, where PIM kinases phosphorylate the BAD at ser 112, which disrupts the association with BCL-2, promoting binding and retention in the cytosol (Yan et al. 2003), hence resulting in anti-apoptotic activity. PIM kinases also inhibit the activation of CASP 3 and 9 (Chen et al. 2009b) (Chen et al. 2009a). The expression of PIM kinases in cancer is sufficient and converges to control the mTOR signaling pathway axis via upstream and downstream effectors (Sengupta et al. 2010); (Beharry et al. 2011). All the three isoforms of PIM kinases were reported to be genes co-activated with *myc* in murine lymphoid tumors (Nawijn et al. 2011). Overexpression of PIM kinases has been reported in a variety of human tumors. PIM1 expression is correlated to aggressive tumors and despite its frequent presence in human cancers, it is considered as weak oncogene. Studies in mice have shown though the incidence of tumor is evident but the oncogenic activity is low. However, the expression of PIM kinases is found to be enhanced in human prostate cancer cell lines (Kim et al. 2010). The most interesting evidence supporting the cooperative property of PIM kinases is explained by their synergism with c-MYC. MYC is a proto-oncogene, which upon overexpression results in apoptosis in normal cells. PIM kinases contribute to tumorigenesis by enhancing MYC regulated signaling pathways. PIM kinases are most evident in myeloid leukemia like acute myeloid leukemia (AML) (Natarajan et al. 2013). PIM is reported to mediate several B cell tumors, including B cell non-Hodgkin's lymphoma (NHL), Burkits lymphoma and multiple myeloma. PIM2 overexpression leads to progression of tumor (Hiasa et al. 2015), PIM and PIM3 kinase are increased in lymphoproliferative diseases associated with Epstein bar virus (EBV). Microarray analyses and immunohistochemical reports have shown that PIM isoforms are elevated in several tumors, including pancreatic (Li und Mukaida 2014), gastric (Yan et al. 2012), renal (Mahalingam et al. 2011), hepatocarcinoma (Leung et al. 2015); (Endo et al. 2007) and colon cancers (Peng et al. 2013).

The PIM1/STAT3 signaling pathway plays a critical role in endothelial differentiation (Iwakura et al. 2011).

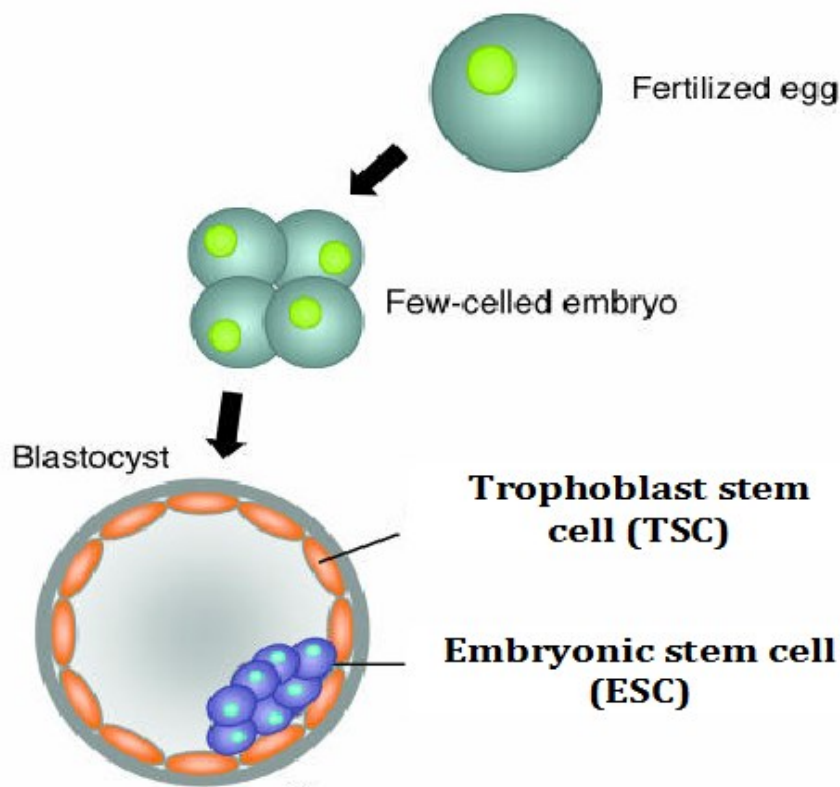


**Figure 11.** Schematic representation of PIM kinases in cell survival and apoptosis.

### 1.13 Stem cells

Discovery of stem cells was a team effort in 1963. Later in the 1980s the research was coined and from there on, it has been a boom to the world of science aiming to solve many diseases and pathologies (Thomson et al. 1998). Stem cells is the undeveloped or undifferentiated biological cell, that can differentiate and divide (mitosis) into new specialized cells which possess the ability of unlimited proliferation, self-renewal and generation of new tissues. The two main types of stem cells are 1. Embryonic stem cells (ESC) and 2. Non-embryonic stem cells. From (Figure. 12) embryonic stem cells have the property of being pluripotent or totipotent due to their nature to differentiate into new cell type. Non-embryonic stem cells (non-ESC) are multi-potent as these cells have a more limited potential to differentiate into various cell types.

ESC have a great ability to spontaneously differentiate and are more prevalent as non-ESC (Thomson et al. 1995); (Thomson und Marshall 1998); (Tuch 2006). ESC cells are obtained from the inner cell mass of blastocysts, which is formed in the initial days of pregnancy, when the blastocyst implants the uterus. These stem cells derived from human blastocyst have normal karyotype, express cell surface markers and have high telomerase activity. Under favorable microenvironment these cells contribute to the formation of three germ layers, namely endoderm, mesoderm and ectoderm. There are two distinct subtypes of blastocyst derived stem cells: 1. ESC and 2. Trophoblast stem cell (TSC). TSC help to study trophoblastic development, which could provide important information for managing pregnancy related pathologies in human (Tanaka et al. 2014).

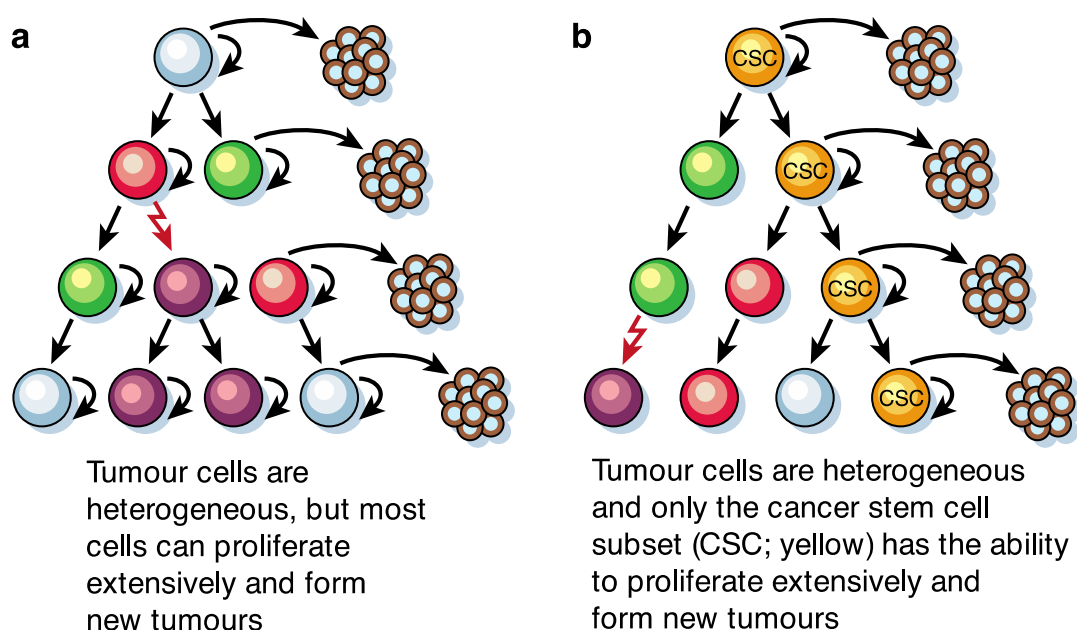


**Figure 12.** Schematic representation of blastocyst derived stem cells

Modified (Landry und Zucker 2004)

### 1.14 Cancer stem cell (CSC)

ESC and TSC can be characterized by the presence of transcription factors responsible for the stemness and by cell surface proteins which support maintaining stem cell properties. It has been reported (Figure. 13) that TSC have the ability to differentiate into embryonic lineages which may enact as tumor cells. (Lansdown und Rees 2011); (Shih le 2011); (Sivasubramaiyan et al. 2009). Cancer stem cells (CSC) were first reported in acute myeloid leukemia (AML) expressing CD34 (Lapidot et al. 1994). They a rare population of cells which has indefinite potential for self-renewal driving tumorigenesis (Reya et al. 2001).



**Figure 13.** Schematic representation of the cell heterogeneity in stem cells

Normal cancer cells and cancer stem cell population, modified (Reya et al. 2001)

During blastocyst implantation and invasion, trophoblast cells differentiate in a fashion mimicking cancerous cells and during cancer progression there is similar behavior of cell differentiation and proliferation as during blastocyst implantation and invasion. CSC is involved as a subpopulation of tumor cell. CSC and TSC are used in research aiming to treat pathologies and diseases which might give valuable information to find novel treatments.

SOX2, Oct-4, NANOG and CDX2 are transcriptional factors essential to maintain self-renewal and undifferentiated stem cells. The expression of surface molecules characterizes stem cells, such as CD44 (multi structural and multifunctional cell surface molecule which is involved in cell differentiation, cell migration, cell proliferation), CD24 (cell adhesion molecule), CD34 (cell adhesion factor and cell migration) and CD133 (involved in stem cell differentiation and cell invasion).

### ***1.15 3D culture-Spheroid***

The 3-dimensional (3D) cell culture system is an artificial environment where biological cells are allowed to grow and interact with their surrounding 3-dimensionally. Living tissue cells exist in 3D microenvironment with cell-cell and cell-matrix communication and complex transport machinery for nutrients for the growth of cells. When cells are grown on non-adherent surfaces they may form spheroid structures that mimic the 3D environment. 3D culture systems reflect the complexities of tissues more realistic than conventional 2D systems (Friedrich et al. 2009).

### ***1.16 PIM kinases -LIF-Stem cell-trophoblast***

As it is stated previously PIM kinases are mainly regulated by the cytokine dependent JAK/STAT pathway. LIF is one of the important factors playing a crucial role during implantation, placentation, trophoblast formation and invasion, migration and proliferation and signals via the JAK/STAT pathway. Pluripotency is supported by PIM kinases (PIM and PIM3) (Aksoy et al. 2007); (Iwakura et al. 2011). PIM3 kinase regulates STAT3 signaling which inhibits cell proliferating in human liver cancers (Wang et al. 2014). PIM kinases are found to support the propagation of mesenchymal stem cells (Zhao et al. 2014) and PIM1 kinase in rejuvenation of human cardiac progenitor cells (Mohsin et al. 2013).

Hence these above mentioned data triggered and lead to the aim of this study.

## **CHAPTER 2 OBJECTIVE**

To characterize PIM kinase functions and the stem cell like properties of trophoblastic cells.

### **2.1 Part I**

To identify PIM kinases in trophoblastic cells.

To analyze the effect of LIF stimulation on trophoblastic cells.

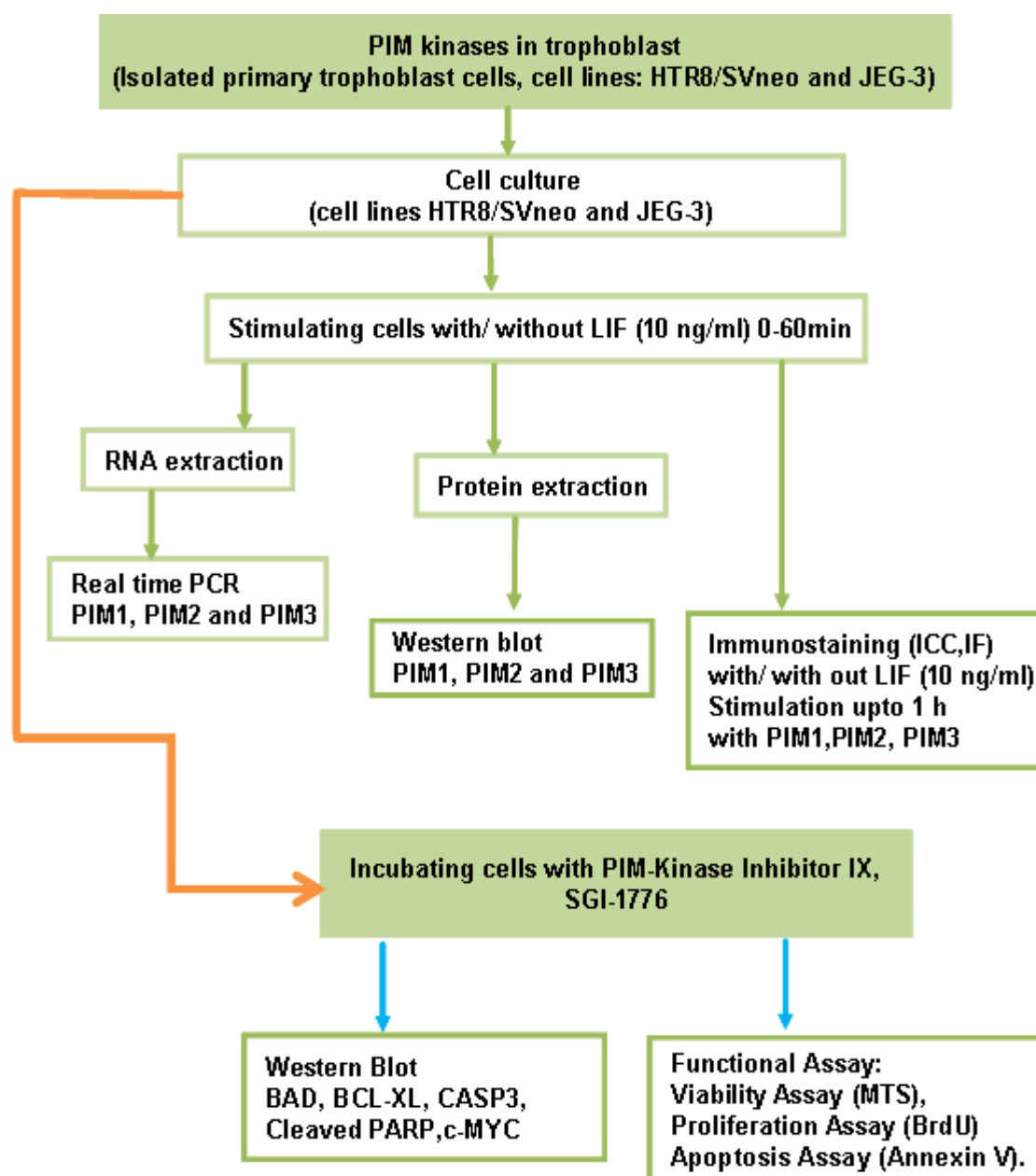
To investigate the functional effects of a chemical inhibitor for PIM kinase.

### **2.2 Part II**

To characterize the spheroids formation of HTR8/SVneo cell that resembles their Cancer stem cell (CSC) like properties.

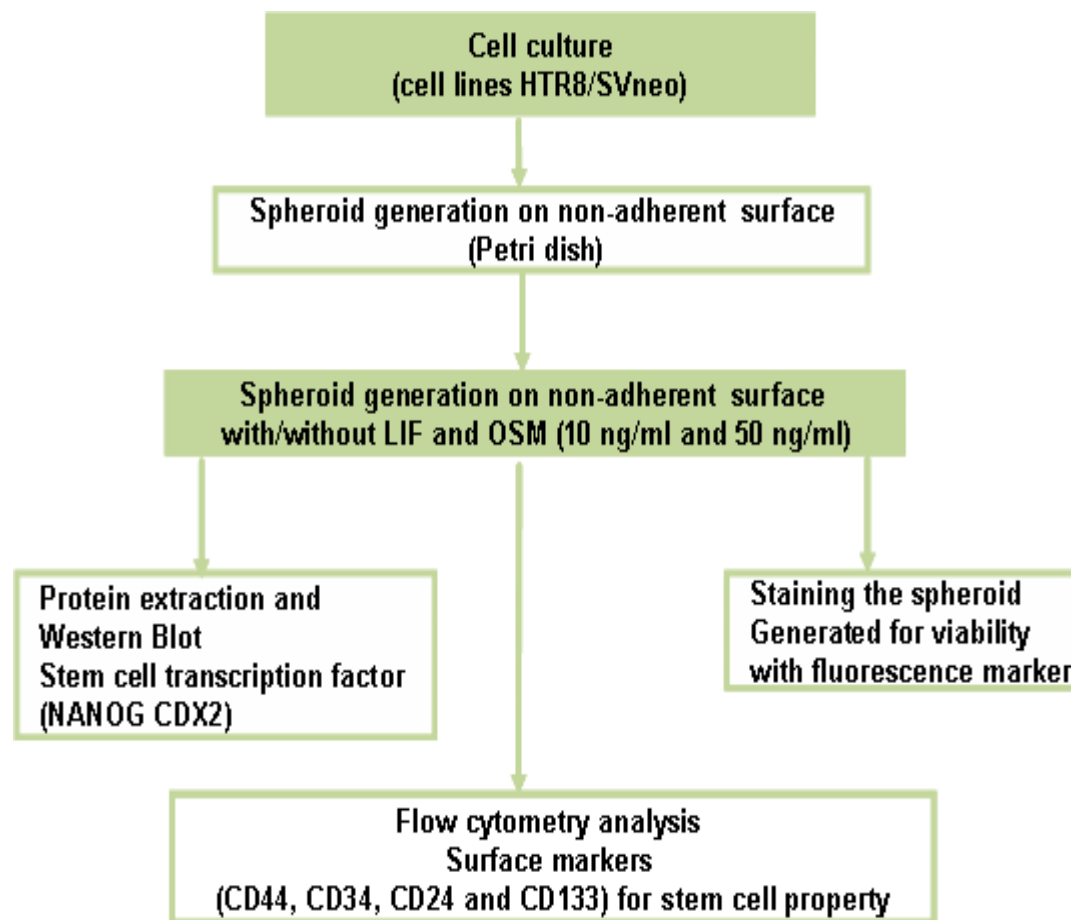
To analyze the effect of cytokines stimulation on the formed spheroids.

## 2.3 Experimental Design Part I





## 2.4 Experimental Design Part II



## CHAPTER 3 MATERIALS AND METHODS

### 3.1. Part I

#### 3.1.1 General Procedure

All investigations were carried out by applying different methods and were performed at least three times. Information regarding each method has been described below, followed by materials and further specific information on individual methods.

#### 3.1.2 Reagents and antibodies or compounds

Antibodies against PIM1, PIM2 and PIM3 kinase, BCL-XL, phospho-BAD, BAD, CASP3, cleaved CASP3, cleaved PARP, phospho-c-MYC, c-MYC, GAPDH, alpha tubulin, and HRP secondary antibody, were purchased from Cell Signaling Technologies (UK). Recombinant Human Leukemia Inhibitory Factor (LIF) was purchased from Merck Millipore (Germany). SGI-1776 a chemical inhibitor for PIM kinases was purchased from Merck Millipore (Germany).

#### 3.1.3 Ethical aspects

Experimental investigation of placentae was performed based on a vote of the local ethics committee at the Friedrich-Schiller University Jena. All analyzed placentae were destined for disposal.

**Table 3.** Culture conditions and Cell line conservation

Cell line	Culture properties	Organism	Morphology	Biosafety level	Origin	Tissue
Primary trophoblast cells	Adherent	Human	Epithelial	2	First or third trimester	Placenta
HTR8/SVneo (GrahamCH,'93)	Adherent	Human	Fibroblast	1	Immortalized primary trophoblast cell	Placenta
JEG-3 (Kohler'70)	Adherent	Human	Epithelial	1	Choriocarcinoma	Placenta

The immortalized human primary trophoblast cell line HTR8/SVneo was a kind gift from Dr. Charles Graham, Ontario, Canada, the choriocarcinoma cell line JEG-3 was obtained from ATCC® HTB-36™. Both cell lines were maintained in the recommended tissue culture media supplemented with 10% fetal bovine serum (FBS) (Sigma Aldrich, Steinheim, Germany), 1000 U/ml penicillin and streptomycin (Life technologies, Grand Island, New York, USA). Cell lines were verified as authentic and were tested for mycoplasma contamination on a quarterly basis using mycoplasma kits (INtRON biotechnology Inc. Sunngam, Korea), which were found to be negative. All cells were grown under standardized conditions (37°C, 5% CO<sub>2</sub>, humidified tissue culture incubator). HTR8/SVneo (monolayer) were cultured in RPMI medium (Life technologies) and JEG-3 (monolayer) was cultured in Hams F12 media (Life technologies).

#### **3.1.4 Isolation of primary trophoblast cells**

The primary trophoblast cells were isolated from term placenta tissue not more than 1 h following collection. The protocol for isolation was adapted and modified from (Kliman et al. 1986) and (Moore et al. 1997) in our Placenta Laboratory. In brief, placenta tissue was cut into small pieces followed by a washing step in sterile 1X PBS to remove blood as much as possible. Tissue was transferred into a new tissue culture flask. The obtained tissue samples were digested enzymatically with a mixture of collagenase, proteases and DNase for 30 min at 37° C. After a washing step the isolated cells were separated by a Percoll gradient (Percoll, Pharmacia, Sweden) and centrifuged at 100 *g* for 10 min. The layer within the 25% Percoll was carefully collected and washed. To avoid contamination RNA lysis buffer was added to the collected isolated cells.

#### **3.1.5 RNA extraction, PCR and quantitative real-time PCR**

Total cellular RNA was extracted using TRIZOL reagent (Invitrogen, Darmstadt, Germany) and quantification of total RNA was determined by Nano Drop ND-1000 spectrophotometer (PeqLab Biotechnologies GmbH, Erlangen, Germany). Samples with purity ratio more than 1.8 at A260/A280 were stored at -80°C. cDNA was generated from 150 ng-300 ng of DNase-free RNA using Maxima Reverse Transcriptase (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA) as per manufactures' instructions. Reverse transcription (RT) was performed using a thermocycler (Eppendorf, Germany).

Amplification reactions were set up in a reaction volume of 20µL by use of Dream Taq DNA Polymerase (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA) and real time quantitative polymerase chain reaction was performed using SYBR green master mix with ROX reference dye (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA) in a Real-time PCR machine Stratagene Mx3005P (Agilent Technologies Inc., Santa clara, CA, USA). PCR primers were self-designed using software tools NCBI, genome browser, Primer3 and the specificity were confirmed using Keinefold and InsilicoPCR UCSC genome browser respectively. The applied concentrations ranged from 0.1 µM up to 1 µM, followed by a gradient PCR and real time PCR. Briefly, HTR8/SVneo and JEG-3 were cultured and seeded in 6 well plates at a density of  $4.5 \times 10^5$  cells/well, followed by incubation until 24 h to reach 80% confluence and cells were stimulated with LIF (10ng/mL). Highly purified salt-free primers for the target genes *PIM1* *PIM2* and *PIM3* (Sigma Aldrich Germany) have been designed and optimized to an equal annealing temperature of 60°C. PCRs were optimized in a Thermo cycler (Master cycler Gradient, Eppendorf, Germany) with regard to Dream Taq.

**Table 4.** Primer Details

Gene Name (PCR Product)	Primer sequence		Gene ID
<b><i>PIM1</i>(109bp)</b>	Fwd	5'CATCCTTATCGACCTCAATCG-3'	NM_001243186. 1
	Rev	5'TATACACTCGGGTCCCATCG-3'	
<b><i>PIM2</i> (107bp)</b>	Fwd	5'TCACAGATCGACTCCAGGTG-3'	NM_006875.3
	Rev	5'CATAGCAGTGCGACTTCGAG-3'	
<b><i>PIM3</i> (128bp)</b>	Fwd	5'ATGCTGCTCTCCAAGTTCGGCTCCCTGGCG-3'	NM_001001852. 3
	Rev	5' TCCTGTGCCGGCTCGGGTCGCTCCAGCACC 3'	
<b><i>HMBS</i> (281bp)</b>	Fwd	5' GAAATCATTGCTATGTCCACCAC 3'	NM_008093.1
	Rev	5' CTTCCCACCACACTCTTCTC 3'	

**Table 5.** Conventional PCR program

Step	Temperature °C.	Time	Number of Cycles
Initial denaturation	95	1 min	1
Denaturation	95	30 s	40
Annealing	T <sub>m</sub> -5	30 s	
Extension	72	1 min	
Final Extension	72	10 min	1

**Table 6.** Real-time PCR program

Step	Temperature °C.	Time	Number of Cycles
Initial denaturation	95	10 min	1
Denaturation	95	15 s	40
Annealing	60	30 s	
Extension	72	30 s	

### 3.1.6 Gel Electrophoresis

Gel electrophoresis was performed to separate the PCR amplicates. According to the fragment size 2% agarose gel electrophoreses were prepared with additional 4  $\mu$ l – “20,000X Red Safe Nucleic Acid staining solution” (final concentration 1X) (INTRON biotechnology Inc. Sungnam, Korea). All PCR amplicates were mixed with 6X orange DNA loading dye (Thermo Fisher scientific, Bioscience, USA) final concentration 1X and filled into the well pockets of the prepared gels. Detection for the fragment length was performed by adding 5  $\mu$ l of the O'GeneRuler 50bp DNA ladder (Thermo Fisher scientific, Bioscience, USA) and loading on the gel.

Amplicates and ladder were separated with electric field of constant voltage 90 V in an electrophoresis chamber (PeqLab Biotechnologies GmbH, Erlangen, Germany) filled with 1X Tris-acetate-EDTA buffer (TAE). All separated DNA fragments were visualized using Ultra violet (UV) light based detection system MF-ChemiBIS 3.2” (Biostep, DNR Bio-Imaging systems, Jerusalem, Israel) and the corresponding "Gel capture" software (Version 5.1).

### 3.1.7 Western blotting

The expression of PIM kinases (PIM1, PIM2 and PIM3) was analyzed by Western blot. Briefly, cells were seeded in 6 well plates at a density of  $4.5 \times 10^5$  cells/well, and each plate was incubated for 24 h to reach adherence and confluence. Subsequently, after serum starvation for 4 h, cells were stimulated with or without LIF (10 ng/ml) from 2 minutes until 60 minutes. Then, cells were rinsed with cold phosphatase buffered saline (PBS; 1X; GE healthcare or PAA, Pasching/Linz, Austria) and harvested. The cells were lysed using Cell lysis buffer (Cell Signaling Technologies Inc., Beverly, Massachusetts) and lysates were centrifuged at 10,000 x g for 10 min at 4° C. Protein concentrations of the resulting supernatant were determined at 660 nm using a Pierce® BCA Protein Assay (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA). Total protein (20-30 $\mu$ g) equivalent concentrations of cell lysate protein were boiled for 7 min at 95° C and resolved in 10-12% SDS PAGE electrophoresis at 60 mA constant for 1 h, followed by protein transfer to a PVDF membrane (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA).

Membranes were blocked with 5 % bovine serum albumin (BSA) in Tris-buffered saline containing 0.1 % Tween 20 (TBST) for 1 h before overnight incubation at 4° C with various primary antibodies: anti-PIM1, anti-PIM2, anti-PIM3, anti-BCLXL, anti-phospho-BAD, anti-BAD, anti-c-MYC, anti-phospho-c-MYC, anti-CASP 3, anti-cleaved CASP 3 and anti-cleaved PARP, typically at 1:1000 dilution. Blots were rinsed with 1X TBST and incubated with a secondary antibody anti-rabbit IgG, HRP-linked (Cell Signaling Technologies, Beverly, Massachusetts) at a dilution 1:10,000 for 90 min at room temperature. Reactive bands were visualized by exposure to Luminata Forte Western HRP substrate (Merck Millipore, Billerica, Massachusetts) a chemical reagent for detection.

Expression was quantified by using the ultra-sensitive chemiluminescence gel documentation system MF-ChemiBIS 3.2" (Biostep, DNR Bio-Imaging systems, Jerusalem, Israel) and densitometry quantification of the respective band intensities was performed using Gel capture Total Lab 1000 image program software.

### **3.1.8 Immunocytochemistry (ICC)**

Immunocytochemistry was performed to confirm the localization and distribution of the expression of PIM kinases in the cell models. HTR8/SVneo and JEG-3 cells were cultured on super frost plus microscope slides (Thermo Scientific, USA) in slides culture chambers (NUNC quadri PERM, Germany). Before seeding the cells , cell culture slides were washed in ethanol (75%) and placed into slide chamber until air dry. 100,000 cells/slides were seeded and incubated overnight followed by LIF (10 ng/ml) stimulation up to 1 h. Staining of cells was performed by using Vectastain Elite rabbit IgG kit (Vector Laboratories, Burlingame, USA). Cells were fixed with methanol/ethanol (1:1) for 5 min followed by washing steps with 1X PBS and permeabilized and blocked using 10% goat serum in 0.1% Tween 20 in PBS for 20 min. Cells were washed 3 times in PBS (1X) (GE healthcare or PAA, Pasching/Linz, Austria) and incubated with 1:100 diluted primary antibodies anti-PIM1, anti-PIM2, anti-PIM3 (Cell Signaling) or rabbit (DA1E) mAb IgG Isotype control (Cell Signaling) in antibody diluent (DAKO, Hamburg, Germany) for 1 h.

Later, the slides with cells were rinsed 3 times with PBS (1X) and incubated with biotinylated affinity-purified anti-rabbit-IgG (Cell Signaling Technologies) for 30 min, followed by treatment with a solution of avidin/Biotinylated enzyme complex (ABC; Thermo Fisher Scientific, Bonn, Germany), followed by 2 min substrate staining with DAB chromogen (DAKO, Hamburg, Germany), followed by washing in distilled water for 2 min and finally, by counterstaining with haematoxylin solution for 1 min. The so prepared slides were dehydrated by ethanol-to-xylene treatment and embedded with Histofluid (Paul Marienfeld, Lauda-Köngshofen, Germany). The images of the slides were visualized on a Zeiss Axioplan2 microscope (Carl Zeiss, Jena, Germany).

### **3.1.9 Immunofluorescence (IF)**

HTR8/SVneo and JEG-3 cells were cultured on super frost plus microscope slides (Thermo scientific, USA). Cell culture slides were washed in ethanol (75%) and placed into a slide chamber until air dry. 100,000 cells/slides were incubated overnight followed by LIF (10ng/ml) stimulation up to 1 h. Staining of cells was performed by using Vectastain Elite rabbit IgG kit (vector Laboratories, Burlingame, USA) where cells were fixed with methanol/ethanol (1:1) for 5 min followed by washing steps with 1X PBS and permeabilization and blocking by using 10% goat serum in 0.1% Tween 20 in PBS for 20 min. Cells were washed 3 times in PBS (1X) (GE healthcare or PAA, Pasching/Linz, Austria) and incubated with the primary antibodies (1:200 dilution) anti-PIM1, anti-PIM2, anti-PIM3 (Cell signaling) or rabbit (DA1E) mAb IgG Isotype control (Cell Signaling Technologies) in antibody diluent (DAKO, Hamburg, Germany) overnight at 4 °C. Later, cells were rinsed 3 times with PBS (1X) and incubated with secondary antibody labeled with fluorescent Cy3 conjugated goat anti-rabbit-IgG (Dianova GmbH, Hamburg, Germany) for 1 h at room temperature, followed by the treatment of the solution with avidin/biotinylated enzyme complex (ABC; Thermo Fisher Scientific, Bonn, Germany), by DAPI (4', 6-diamidino-2-phenylindole) mounting media staining and embedding. The images of the slides were visualized on a Zeiss Axioplan2 microscope (Carl Zeiss, Jena, Germany).



### 3.1.10 Incubation of cells with PIM-Kinase Inhibitor IX, SGI-1776

SGI-1776 {N-((1-Methylpiperidin-4-yl)methyl)-3-(3(trifluoromethoxy)phenyl)imidazo[1,2-b]pyridazin-6-amine, 2H<sub>2</sub>SO<sub>4</sub>AS (Calbiochem®, Millipore Merck, Darmstadt, Germany) is a chemical inhibitor for PIM kinases which is ATP competitive inhibitor of PIM1,2,3 and had to be resolved in dimethylsulfoxide (DMSO) (Sigma Aldrich, Steinheim, Germany). The respective DMSO concentration in medium served as negative control. 500.000 cells/well were seeded in 6 well plates.

After 24 h, cells were treated with either PIM1 kinase inhibitor SGI-1776 or DMSO (0.01%) control vehicle reagents. After incubation for further 24 h protein was extracted for Western blotting.

### 3.1.11 Cell Viability Assay (MTS)

Viable cells were evaluated using a colorimetric MTS (3-(4, 5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) CellTiter 96® AQueous One Solution Cell Proliferation Assay (MTS) (Promega, USA). Cells were seeded at a density of  $5 \times 10^3$  cells/well in triplicates in 96-well plates with outer wells left for blank. After 24 h of incubation, the media was changed and 0.01% DMSO vehicle, or SGI-1776 at different concentrations (5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M) were added and incubated. After further 24 h, 0.5 mg/ml MTS dye was added and the cells were incubated for an additional 4 h prior to measurement. Formazan crystals were dissolved in DMSO for 5 min and the plates were read at 490 nm wavelength spectrophotometrically with a reference of 650nm.

### 3.1.12 Cell Proliferation (BrdU)

Cell proliferation was quantified using 5'-bromo-2'-deoxyuridine (BrdU) incorporation, a Cell Proliferation ELISA, BrdU (Colometric) kit (Roche Diagnostics GmbH, Mannheim, Germany). It is based on the detection of BrdU incorporated into genomic DNA of proliferating cells. Cells were seeded at a density of  $5 \times 10^3$  cells/well (in a final volume of 100  $\mu$ l/well) in triplicates in 96-well plates with outer wells left for distilled water. After 24 h of incubation, the media was changed followed by incubating the cells with or without SGI-1776 at different concentrations (5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M) or 0.01% DMSO vehicle for 24 h at 37 °C at 5% CO<sub>2</sub>. Briefly, cells were labeled with BrdU and allowed to accrue within proliferation cells for 2 h.

Next step, cells were fixed by using FixDenate for 30 min at room temperature followed by incubating with an anti-BrdU-POD monoclonal antibody for 90 min.

Later, the antibody conjugate was removed by flicking off and washing the wells three times with washing solution PBS 1X. Finally, substrate solution was added and incubated until color development was sufficient. Absorbance was measured at 492 nm.

### **3.1.13 Apoptosis (Annexin V assay)**

Apoptosis was measured by flow cytometry using an annexin V FITC conjugated (Immuno Tools, Freisoythe, Germany) and Propidium Iodide (PI) apoptosis kit (BD Bioscience USA) according to manufacturers' instructions. Briefly, cells were seeded in 6 well plates at a density of  $4.5 \times 10^5$  cells/well, and incubated for 24 h to reach adherence and confluence, followed by incubation with or without SGI-1776 at different concentrations (5  $\mu$ M, 10  $\mu$ M and 20  $\mu$ M) or and 0.01% DMSO vehicle. After further incubation for 24 h at 37 ° C at 5% CO<sub>2</sub>, cells were harvested, washed with cold PBS (1X) and re-suspended in annexin V FITC conjugated (which binds extra-cellularity to apoptotic cells)/ Propidium iodide (PI; which stains necrotic cells) for 15mins in dark at room temperature. Fluorescence of  $10^4$  labeled cells was measured by flow cytometry using a FACSCalibur™ (BD Bioscience USA). Viable cells were quantified by subtracting the apoptotic (Annexin V-FITC positive) and necrotic fractions (PI positive).

## **3.2 PART II**

### **3.2.1 Cell cultures and cell line**

The immortalized human primary trophoblast cell line HTR8/SVneo was used for experiments. The cell line was maintained under standardized conditions (37°C, 5% CO<sub>2</sub>, humidified atmosphere). HTR8/SVneo cells were cultured in RPMI medium supplemented with 10% fetal bovine serum (FBS) and 1000 U/ml Penicillin and streptomycin.

### **3.2.2 Generation of spheroids**

Cells were cultured and seeded on non-adherent petri dishes (Greiner, Germany) at a density of  $1 \times 10^6$  cells/petri dish, incubated for 72 h (humidified atmosphere, at 37 °C and 5% CO<sub>2</sub>). Subsequently, spheroids were formed.

### 3.2.3 Viability analysis of formed spheroids

The spheroid viability was analyzed by application of cell tracker dyes, which stain living cells (Cell Tracker™ Probes for long-term tracing of living cells: Cell Tracker™ Green BODIPY; 8-chloromethyl-4, 4-difluoro-1, 3, 5, 7-tetramethyl-4-bora-3a, 4a-diaza-s-indacene; Life Technologies, Germany). Spheroids formed on 5<sup>th</sup> to 7<sup>th</sup> day were taken for analysis after centrifugation and aspiration of the supernatant. The spheroids were gently re-suspended in pre-warmed Cell Tracker™ dye working solution and incubated for 15–45 min. Subsequently, cells were centrifuged at 1300 rpm for 5 min. The dye working solution was replaced with fresh, pre-warmed medium and cells were incubated for another 30 minutes at 37°C and 5% CO<sub>2</sub> and observed under microscope.

### 3.2.4 Spheroid Stimulation

Spheroids were formed under simultaneous stimulation with 10 and 50 ng/ml human recombinant LIF (Millipore Merck, Germany) or human recombinant OSM (Life Technologies, Germany) or without as control.

### 3.2.5 Flow Cytometry

The HTR8/SVneo cells cultured as monolayers or spheroids were stimulated with LIF or OSM (10 ng/ml and 50 ng/ml). After 72 h, the monolayer cells and spheroids were stained with fluorescence labeled antibodies against surface molecules CD44 (multi structural and multifunctional molecule involved in cell differentiation, migration, and proliferation), CD34 (adhesion molecule, migration and invasion), CD24 (cell adhesion molecule) and CD133 (involved in stem cell differentiation and cell invasion) (all from Miltenyi Biotech GmbH Bergisch Gladbach, Germany) and incubated at 4 °C in a shaker Orbital Taumler Plymax 1040 (Heidolph Instruments GmbH & Co. KG, Schwabach, Germany). After 30 min, cells were washed in PBS 1X, resuspended in FACS Flow buffer (BD, Bioscience, USA) and analyzed on a flow cytometry FACS Calibur (BD, Bioscience, USA).

### 3.2.6 Western Blot

The expression of CDX2, SOX2, OCT4 and NANOG, which are transcriptional factors essential to maintain self-renewal and the status of undifferentiated embryonic stem cells, was analyzed by Western blotting.

The formed spheroids were harvested and rinsed with cold PBS (1X) (GE healthcare or PAA, Pasching/Linz, Austria) and harvested. Spheroids were lysed using Cell lysis buffer (Cell Signaling Technologies Inc., Beverly, Massachusetts) and cell lysates were centrifuged at 10,000 x g for 10 min at 4°C. Protein concentrations of the resulting supernatant were determined by a Pierce® BCA Protein Assay (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA) at 660 nm wavelength. Total protein (20-30 µg) equivalent concentrations of cell lysate protein were boiled for 7 min at 95°C and resolved in 10-12% SDS PAGE electrophoresis at 60 mA constant for 1 h, followed by protein transfer to PVDF membrane (Thermo Fisher Scientific Biosciences GmbH, Waltham, MA, USA). Membranes were blocked with 5 % bovine serum albumin (BSA) in Tris-buffered saline containing 0.1 % Tween 20 (TBST) for 1 h before overnight incubation at 4°C with primary antibodies against CDX2, NANOG, OCT4A, and SOX2, typically at 1:1000 dilutions. Blots were rinsed with 1X TBST and incubated with a secondary HRP-linked anti-rabbit IgG antibody (Cell Signaling Technologies Inc., Beverly, Massachusetts) at a dilution 1:10,000 for 90 min at room temperature. Reactive bands were visualized by exposure to Luminata Forte Western HRP substrate (Merck Millipore, Billerica, Massachusetts) a chemical reagent for detection. Expression was quantified by using the ultra-sensitive chemiluminescence gel documentation system MF ChemiBIS 3.2 (Biostep DNR Bio-Imaging systems, Jerusalem, Israel) and densitometry quantification of the respective band intensities was performed using Total Lab 1000 image program software.

### 3.3 Statistics

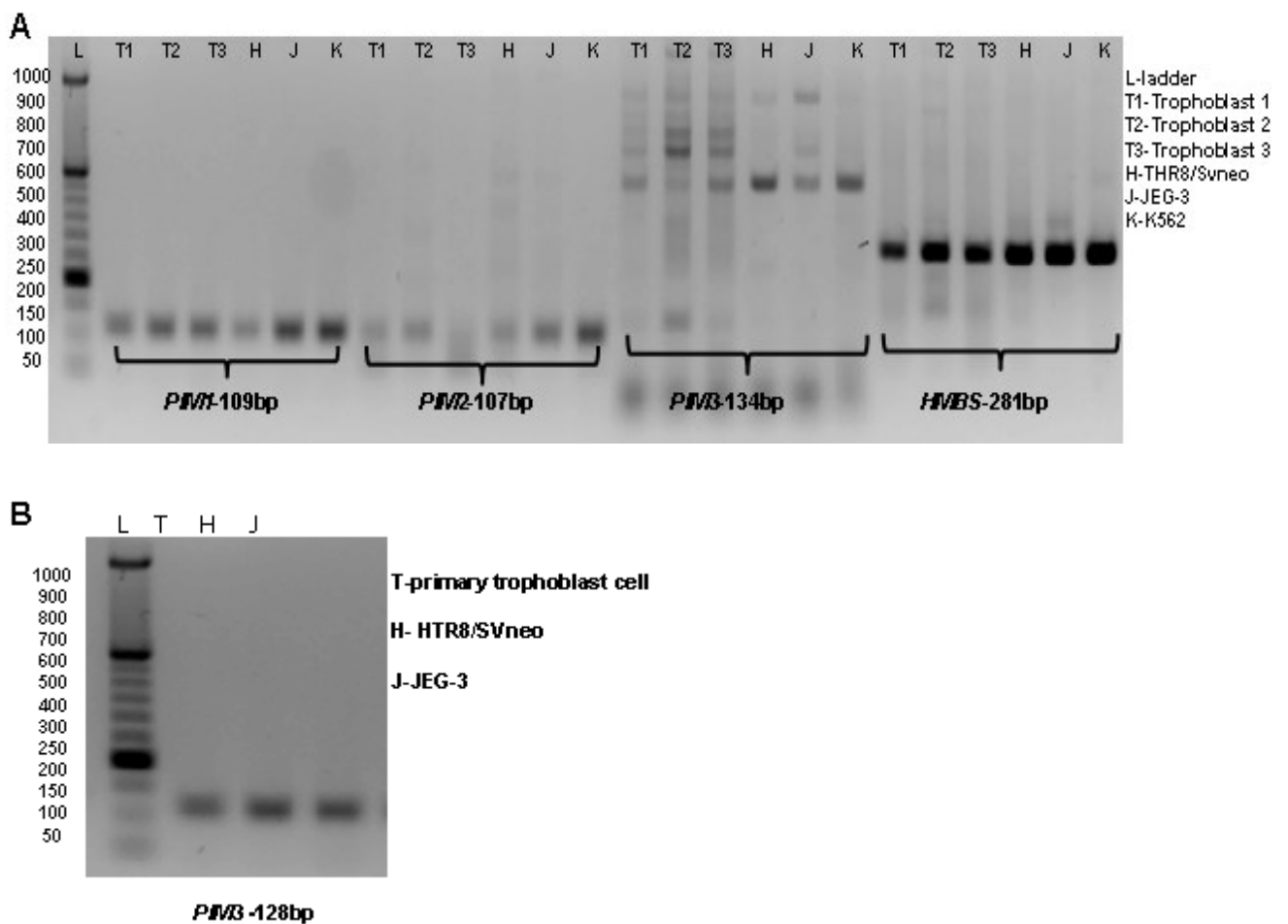
All experiments were repeated at least three times. Data is presented as mean ± Standard Deviation (SD). The quantification was done using delta delta cycle threshold ( $\Delta\Delta C_t$ ) method. Two-tailed Student's t-test was performed for statistical comparisons between experimental groups.  $P < 0.05$  was defined as statistically significant.

## CHAPTER 4 RESULTS

### PART I

#### 4.1 Identification of PIM kinases (PIM1, PIM2 and PIM3) in trophoblastic cells

All three PIM kinase family members were expressed at the mRNA level in primary trophoblast cells, HTR8/SVneo (immortalized transformed trophoblast cells) and JEG-3 (choriocarcinoma) cells (Fig 14.). Shown is the PCR agarose gel electrophoresis.



**Figure 14.** PCR gel electrophoresis PIM kinase expression

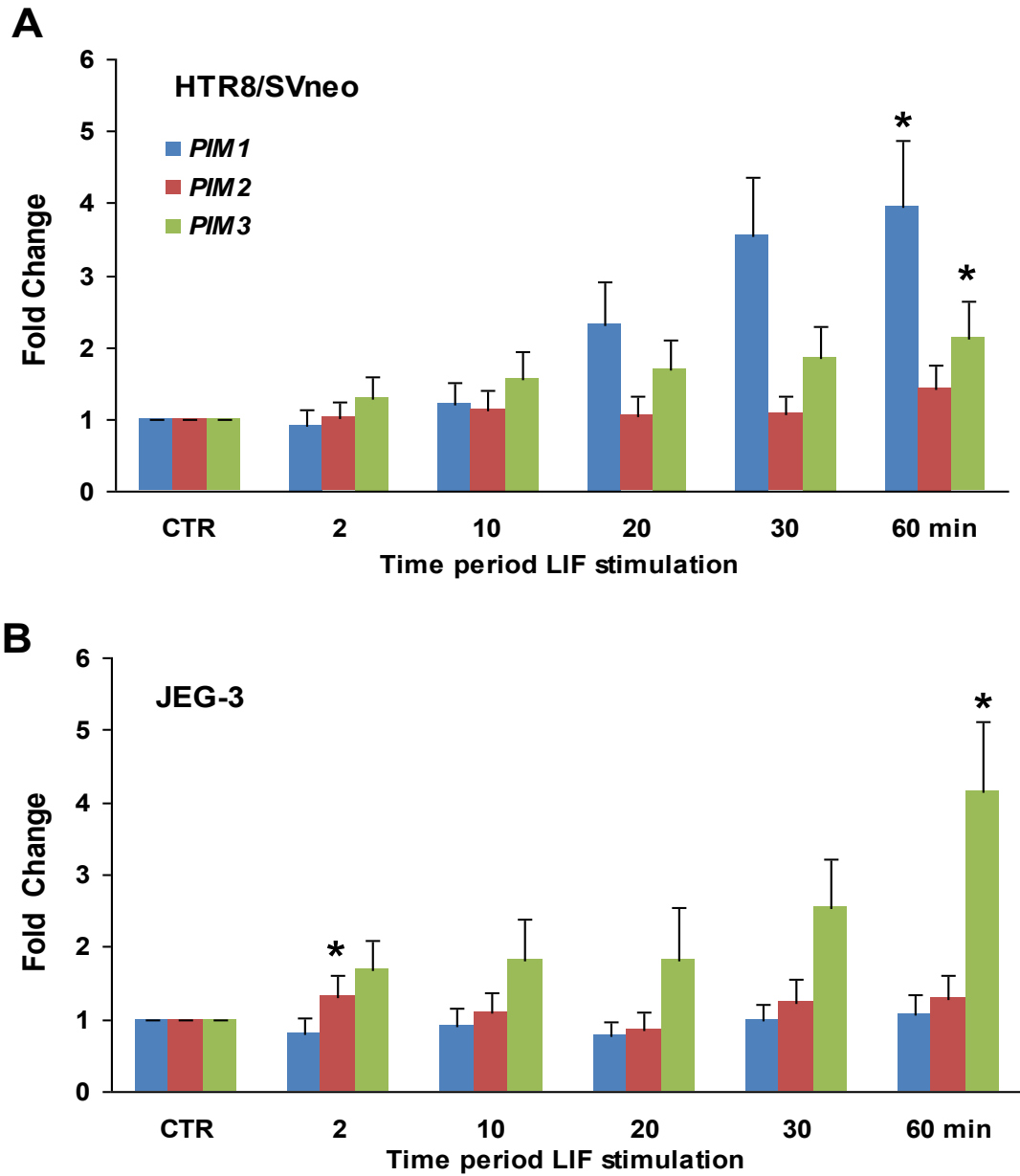
(A) expression PIM kinase in primary trophoblast cells (T1-T3), HTR8/SVneo, JEG-3 and K562 (Leukemic) cells.(B) PIM3 kinase re-designed primer.

## 4.2 Effect of LIF upon stimulation in trophoblastic cells

During pregnancy LIF plays a very important role in trophoblast functions, hence we have stimulated the cell lines (HTR8/SVneo and JEG-3) with LIF (10 ng/ml) from 2'-60' min and studied the expression level of PIM kinase family members (PIM1, PIM2 and PIM3), by real-time quantitative polymerase chain reaction (qPCR) and Western blot analysis. (n=3).

### 4.2.1 Gene expression level

In HTR8/SVneo cells, the gene expression level of *PIM1* and *PIM3* kinase was significantly increased 60 min after LIF stimulation whereas *PIM2* kinase was not significantly altered (Fig 15.A). In JEG-3 cells the gene expression levels of *PIM2* (2 min after LIF stimulation) and *PIM3* kinase (60 min after LIF stimulation) exhibits significant increase but not that of *PIM1* kinase (Fig 15.B). The fold changed was observed by normalization to reference gene *HMBS*. K562 was not positive for all PIM kinase so it was not further used in this study.

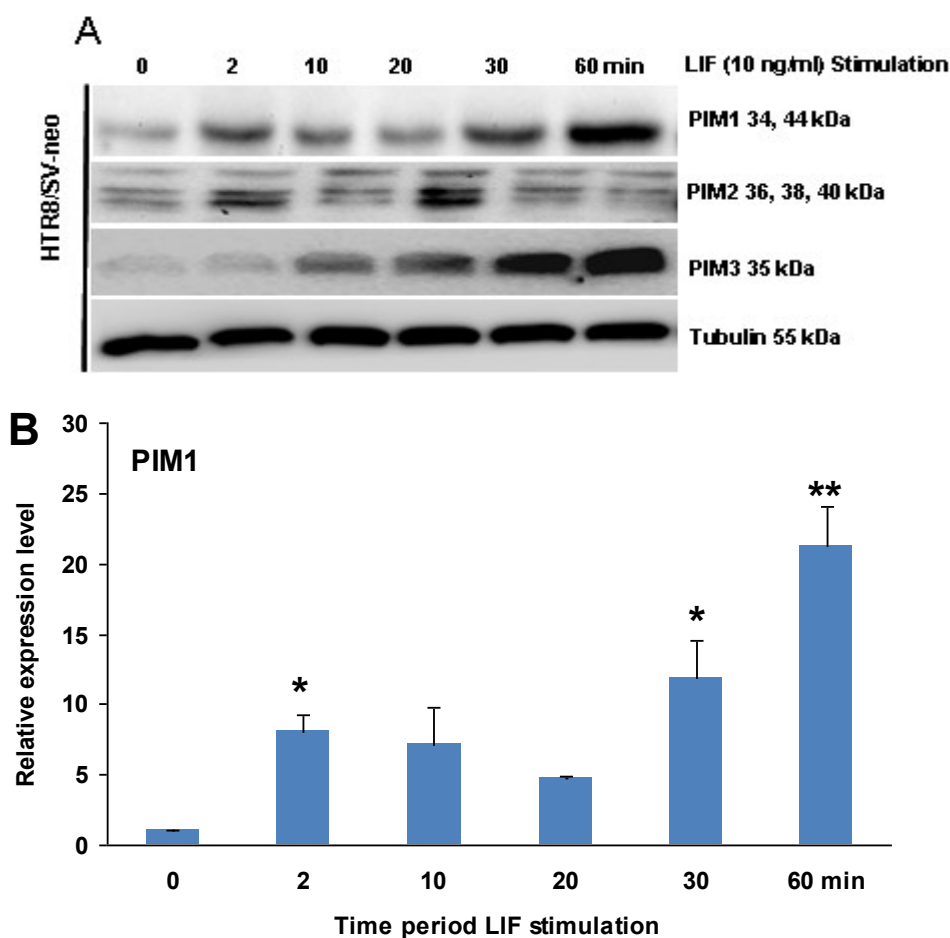


**Figure 15.** Fold change in the expression of PIM Kinases

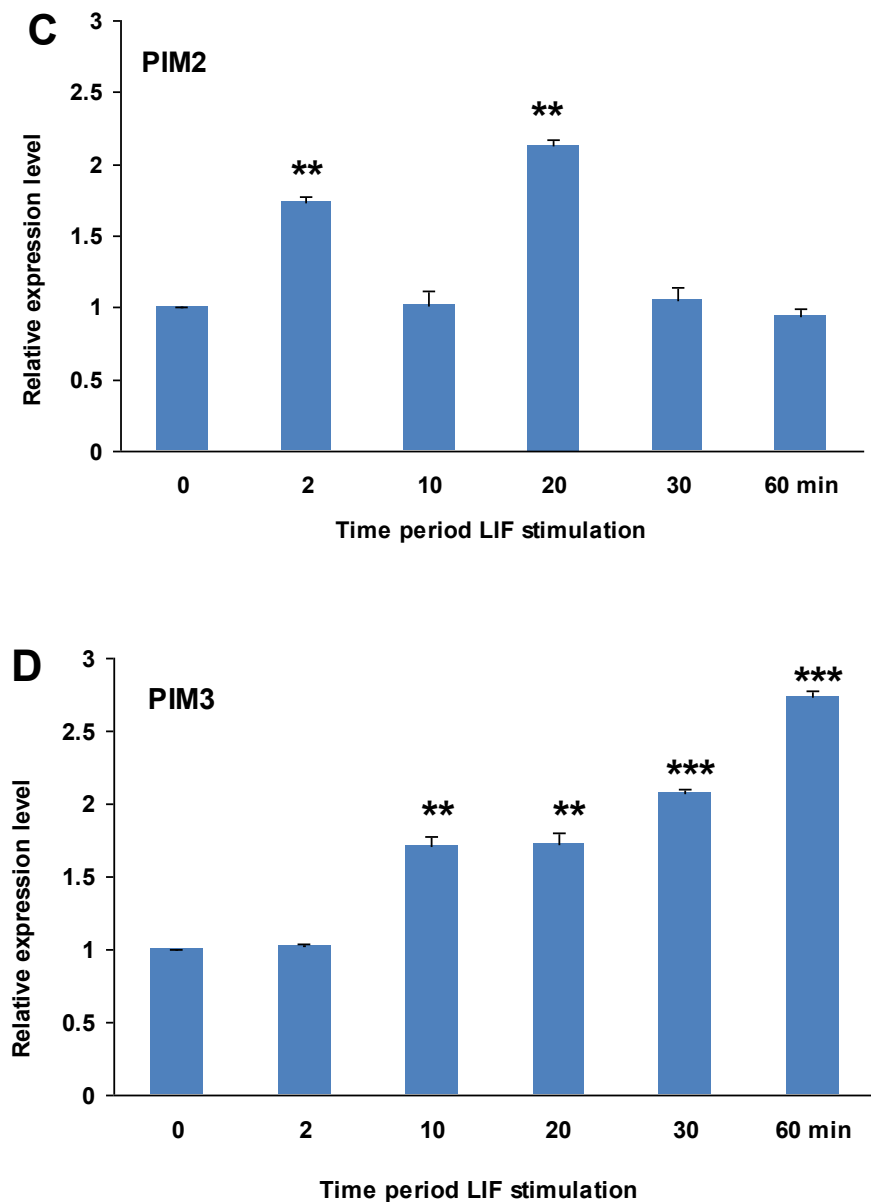
PIM1, PIM2, and PIM3 in HTR8/SVneo (A) and JEG-3 cells (B). Cells were stimulated with LIF (10ng/ml) (n=3-independent experiments, Mean  $\pm$  SD \*  $P < 0.05$ ).

### 4.2.2 Protein expression level

The protein synthesis of PIM kinases family members upon LIF stimulation was analyzed by Western blotting. As above the cells were stimulated, followed by protein isolation, quantification and separation of proteins in SDS-PAGE gel. The proteins were transferred onto membranes, incubated with specific antibodies and the respective bands were visualized by chemiluminescence. Immunoblots of HTR8/SVneo (Fig.16.A) and JEG-3 (Fig.17.A) are shown below. After LIF stimulation, in HTR8/SVneo cells the relative expression of PIM1 (Fig.16. B) and PIM3 (Fig.16. D) Kinases were significantly increased. However, PIM2 kinase showed an increase during the first 20 min followed by a decrease (Fig.16.C). In JEG-3 cells, the relative expression level of PIM2 (Fig.17. C) and PIM3 (Fig.17.D) kinase were significantly increased, but PIM1 kinase was decreased upon LIF stimulation (Fig.17.B). The relative expression was quantified by densitometry and normalized to reference protein alpha-tubulin in all protein analysis experiments.

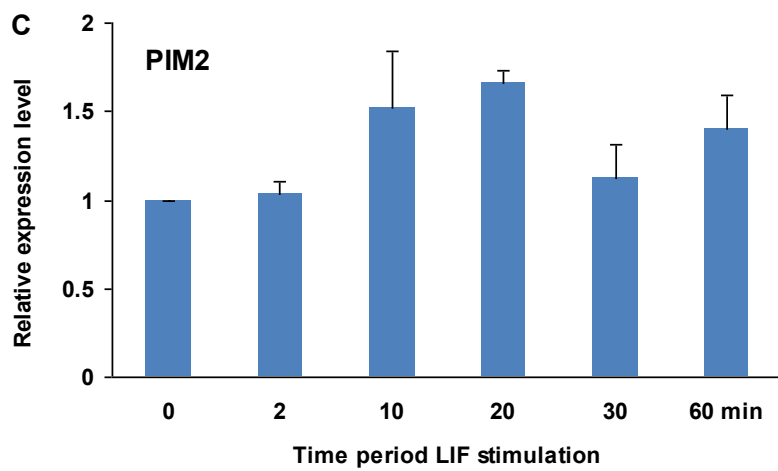
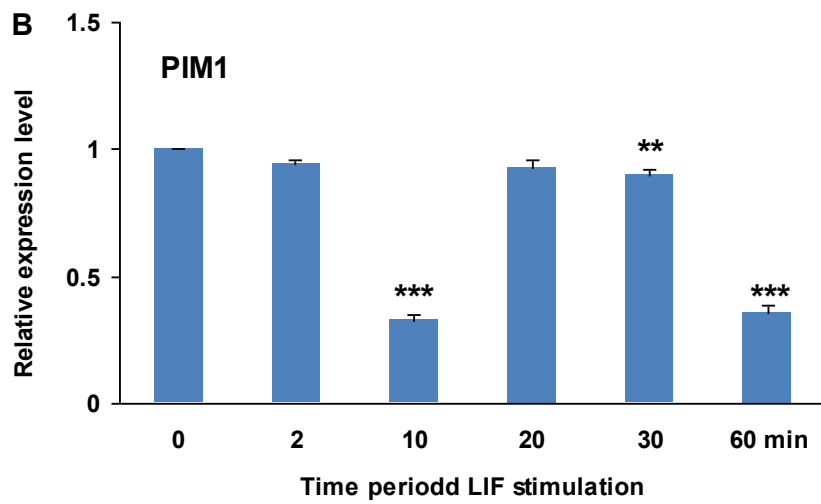
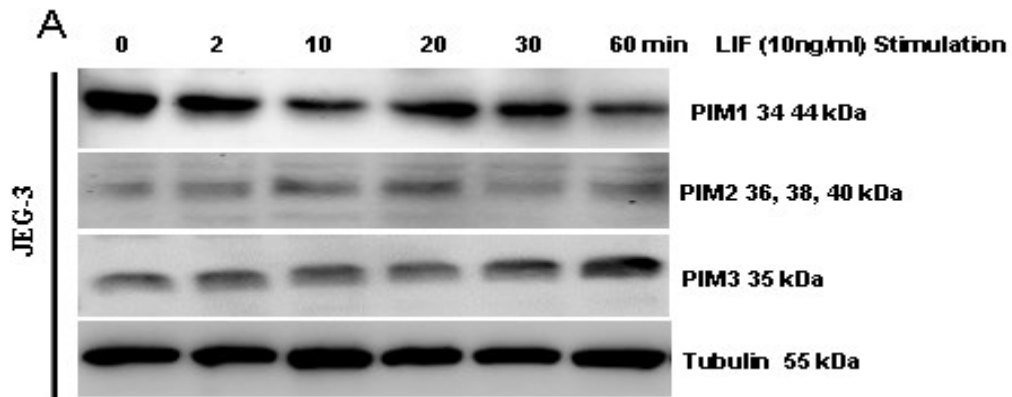


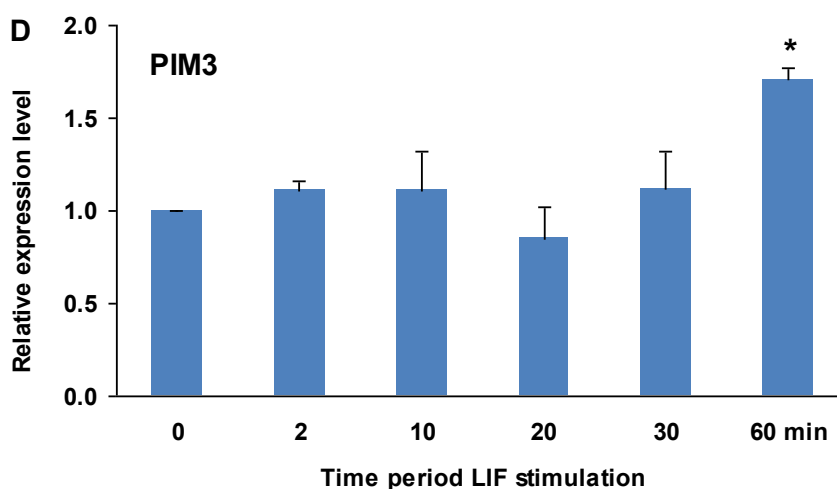




**Figure 16.** Protein expression in HTR8/SVneo cells.

(A) Representative Immunoblots of the protein expression of PIM1, PIM2, and PIM3 upon LIF stimulation in HTR8/SVneo cells. (B, C and D) Densitometry analysis of the relative expression of PIM1, PIM2, and PIM3 kinases in HTR8/SVneo cells. PIM1 and PIM3 kinase expression in HTR8/SVneo cells shows an increase, whereas PIM2 was increased for short duration, later decreased (\*  $p < 0.05$ ;  $p < 0.01$ ;  $p < 0.001$  Student's t-test). Cells were stimulated with LIF (10 ng/ml). Normalized to loading control alpha-tubulin.  $n=3$ - independent experiments, Mean  $\pm$  SD



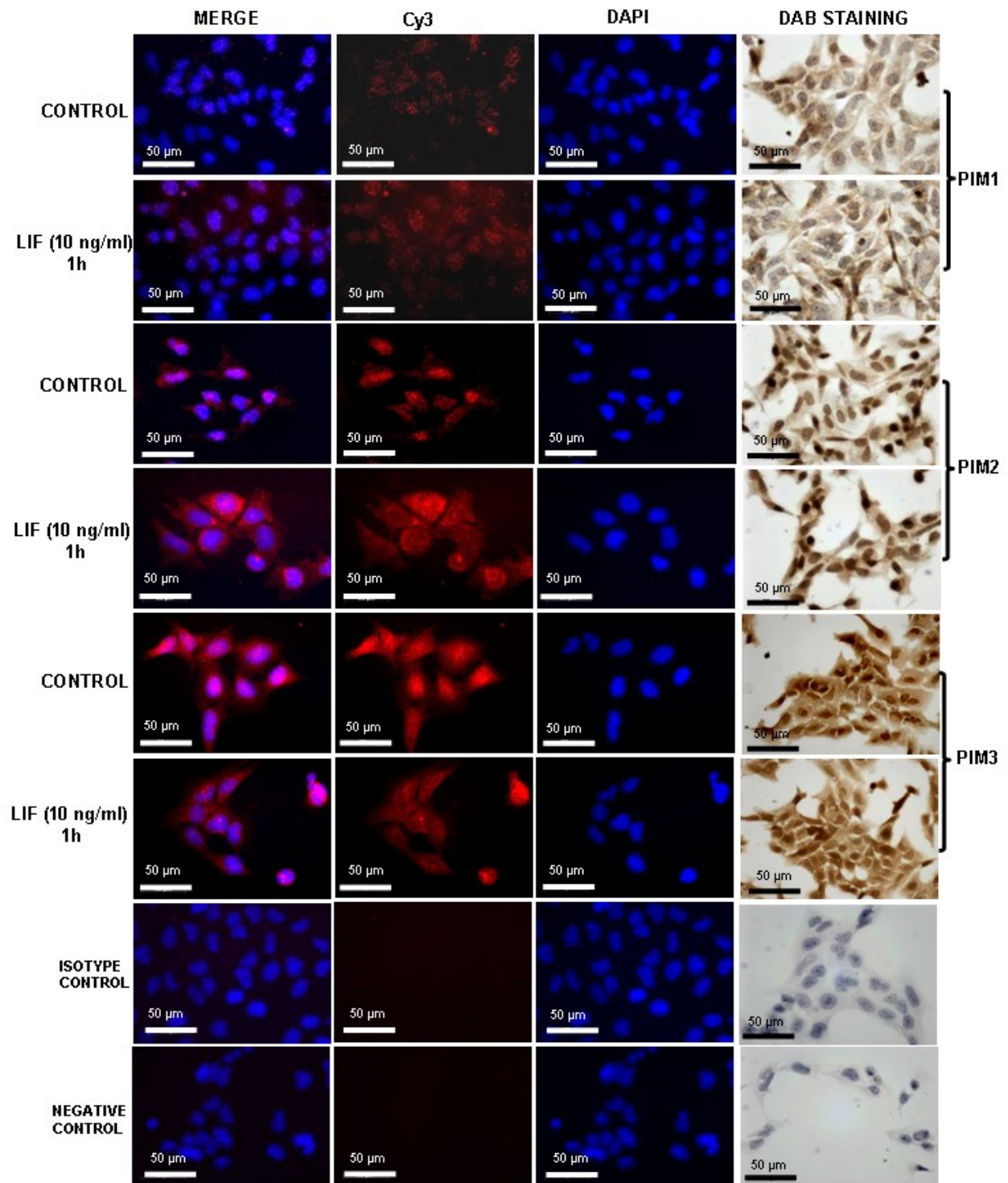


**Figure 17.** Protein expression in JEG-3 cells.

(A) Representative Immunoblots of the protein expression of PIM1, PIM2, and PIM3 with tubulin as reference control upon LIF stimulation in JEG-3 cells. (B, C and D) Densitometry analysis of the relative expression of PIM1, PIM2, and PIM3 in JEG-3 cells. PIM2 and PIM3 kinase expression in JEG-3 cells shows an increase, whereas PIM1 was relatively decreased (\* $p < 0.05$ ;  $p < 0.01$ ;  $p < 0.001$ ; Student's t-test). Cells were stimulated with LIF (10ng/ml). Normalized to loading control alpha-tubulin. N=3- independent experiments, Mean  $\pm$  SD.

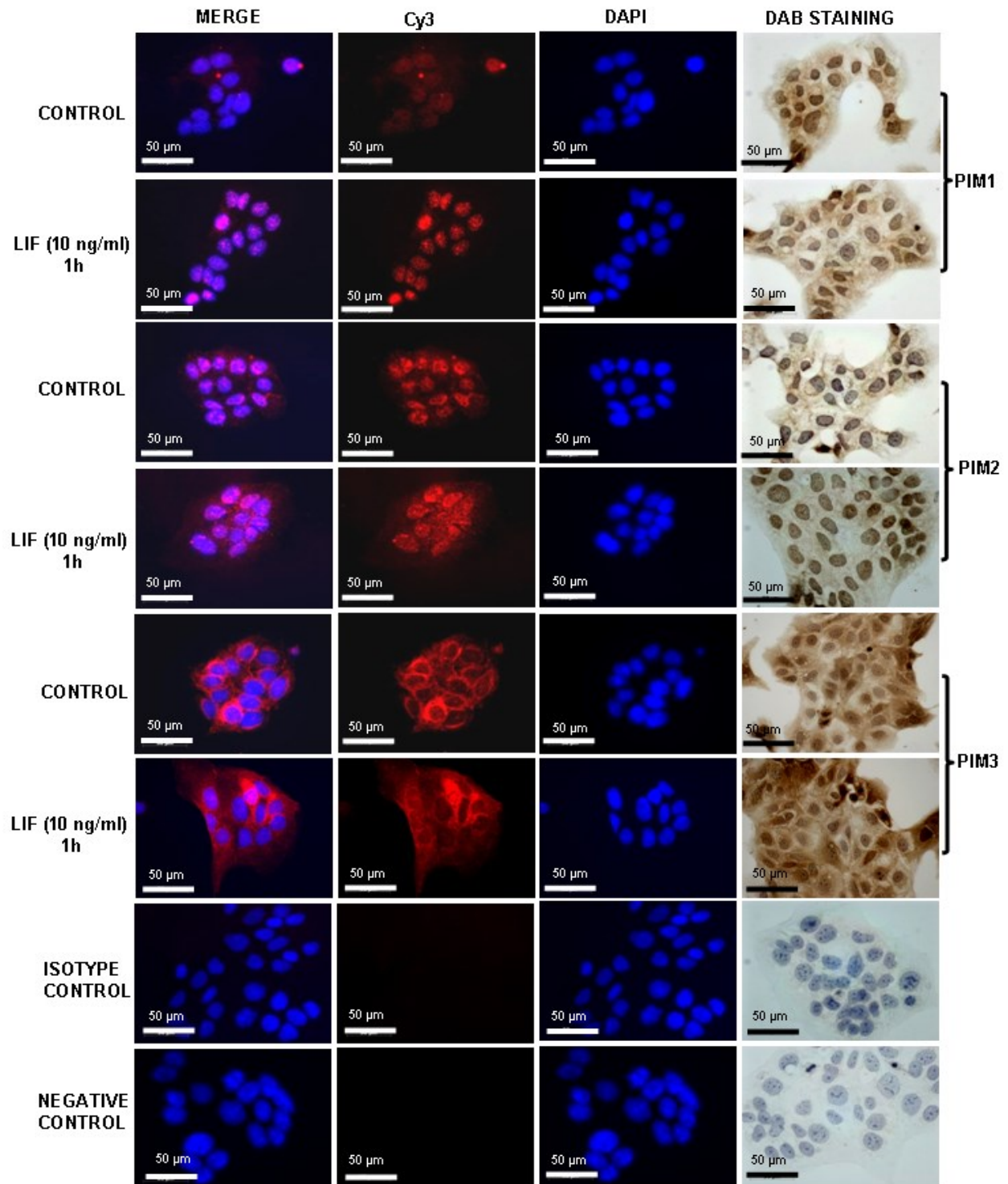
#### 4.2.3 Localization and distribution of PIM kinases

Immunostaining was performed to investigate the morphological localization and distribution of PIM kinases in cell lines. Both immunocytochemistry (ICC) and immunofluorescence (IF) staining were used to study the expression of PIM1, PIM2 and PIM3 kinases upon LIF stimulation up to 1 h. Below figures represent the morphological distributions of PIM1 kinase. In unstimulated cells, PIM1 kinase was localized in cytoplasmic, PIM2 in the nucleus and PIM3 was present all over the cells. Upon LIF stimulation, in HTR8/SVneo (Fig. 18) PIM1 and PIM2 kinase has evidently increase whereas PIM3 kinase is decreased distribution with respect to Isotype control and unstimulated. In JEG-3, (Fig. 19) PIM1 and PIM3 (evidently increased) whereas PIM2 kinase is not evidently increase after LIF stimulation.



**Figure 18.** Immune staining in HTR8/SVneo cells.

IF (From left three columns, merge; nuclei: DAPI; blue, Cy3: red) and ICC (right column, DAB staining -brown) staining showing expression of PIM1, PIM2 and PIM3 kinases in HTR8/SVneo cells. PIM1 and PIM2 show an increase, PIM3 show decrease upon stimulation with LIF (10 ng/ml, 1 h).



**Figure 19.** Immune staining in JEG-3 cells

IF (From left three columns, merge; nuclei: DAPI; blue, Cy3: red) and ICC (right column, DAB staining -brown) staining showing expression of PIM1, PIM2 and PIM3 in JEG-3 cells. PIM1, PIM2 and PIM3 shows an increase compared upon stimulation with LIF (10 ng/ml, 1 h).

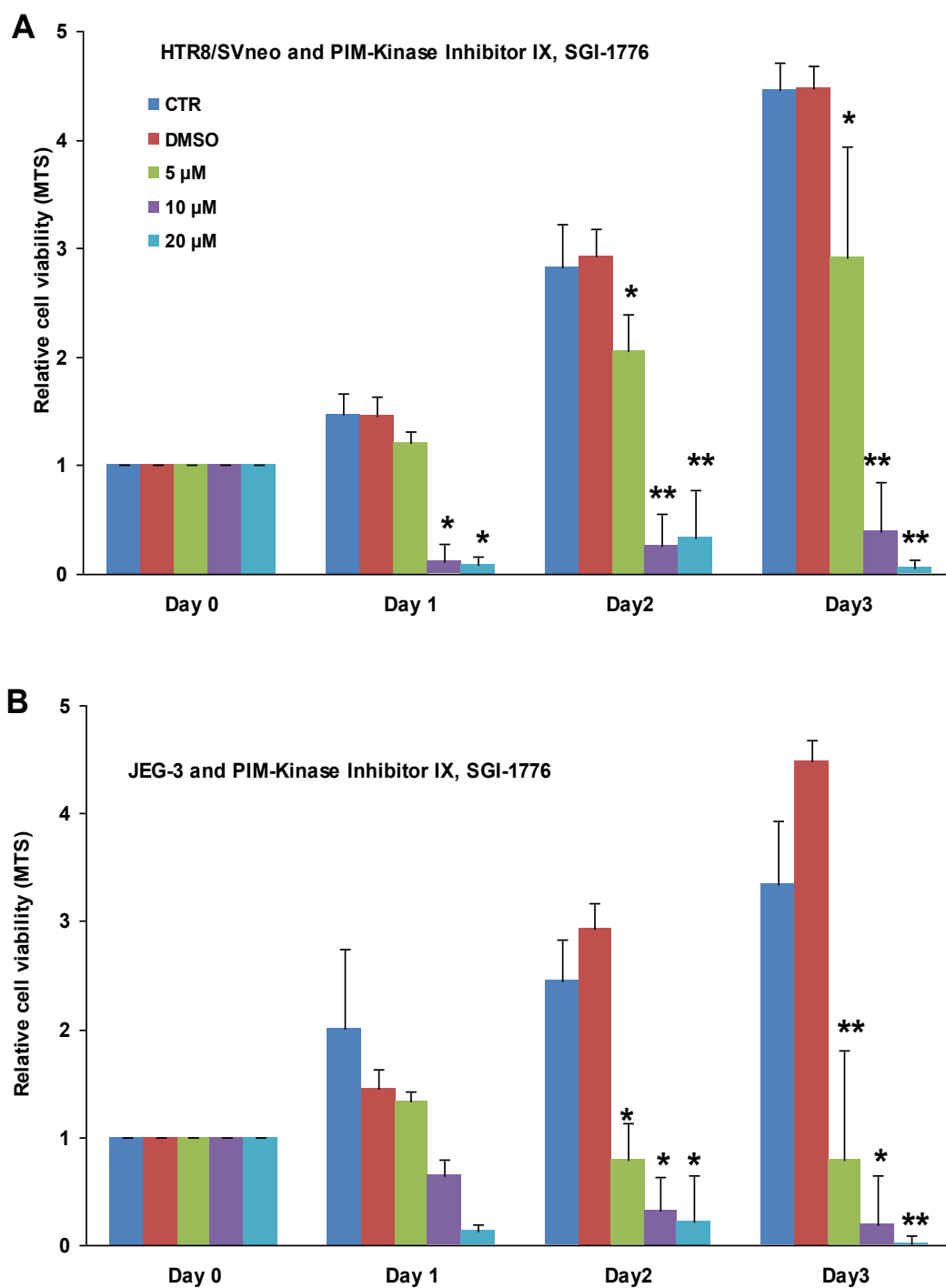
### **4.3 PIM kinase inhibition in trophoblastic cells by SGI-1776**

Cells were incubated with chemical inhibitor, PIM-Kinase Inhibitor IX, SGI-1776; used to inhibit the PIM kinases activity. The functional investigation shows the effect of SGI-1776 on cell viability and proliferation. (n=3)

#### **4.3.1 Effect on Cell viability and proliferation**

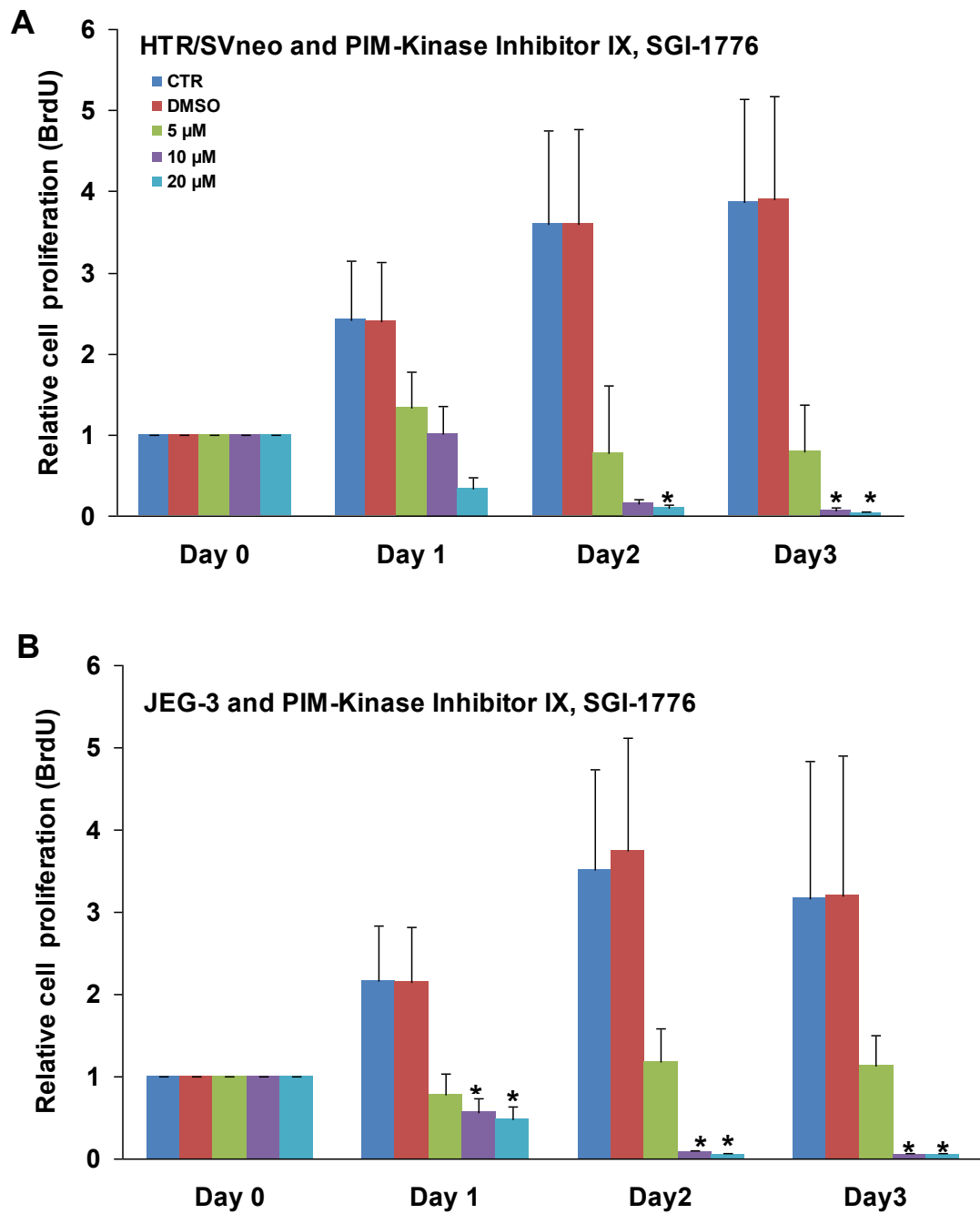
The viability of cells upon SGI-1776 inhibition decreased in dose dependent manner with respect to controls. On day 1 and 2, there was significant reduction of viability at 10 and 20  $\mu$ M SGI-1776. On day 3 at 20 $\mu$ M there were no viable cells in neither cell line. In HTR8/SVneo cells (Fig. 20 A) there was significant decrease in proliferation on day 1 at 20  $\mu$ M SGI-1776, on day 2 and day 3 at 10 and 20 $\mu$ M. In JEG-3 cells (Fig. 20 B) on day 1 at concentrations of 10 and 20 $\mu$ M SGI-1776 decreased viability on day 1 and which was almost completely inhibited on days 2 and day 3. Hence the above data demonstrate that PIM kinases inhibition reduces the cell viability.

The incorporation of BrdU showed similar effect as that of viability where, in HTR8/SVneo cells (Fig. 21 A) on day 1 there is reduction in proliferation rate of cells. On days 2 and 3 showed significant decreased cell proliferation in dose dependent manner. In JEG-3 cells (Fig. 21 A) on day 1, day 2 and day 3 has decreased cell proliferation at 10 and 20  $\mu$ M/ml concentrations respectively.



**Figure 20.** Cell Viability assay

Effect of PIM-kinase inhibitor IX, SGI-1776 (chemical inhibitor) on cell viability (MTS assay) in HTR8/SVneo (A) and JEG-3 cells (B), that were untreated, treated with 0.1% DMSO vehicle alone, or with 5 µM, 10 µM, or 20 µM for 24 h, 48 h or 72 h (n=3 independent experiments, mean ± SD; \*p< 0.05, Student's t-test).



**Figure 21.** Cell Proliferation assay

Effect of PIM-kinase inhibitor IX, SGI-1776 (chemical inhibitor) on cell proliferation (BrdU) in HTR8/SVneo (A) and JEG-3 cells (B), that were untreated, treated with 0.1% DMSO vehicle alone, or with 5  $\mu$ M, 10  $\mu$ M, or 20  $\mu$ M for 24 h, 48 h or 72 h (n=3 independent experiments, mean  $\pm$  SD; \*p< 0.05, Student's t-test).



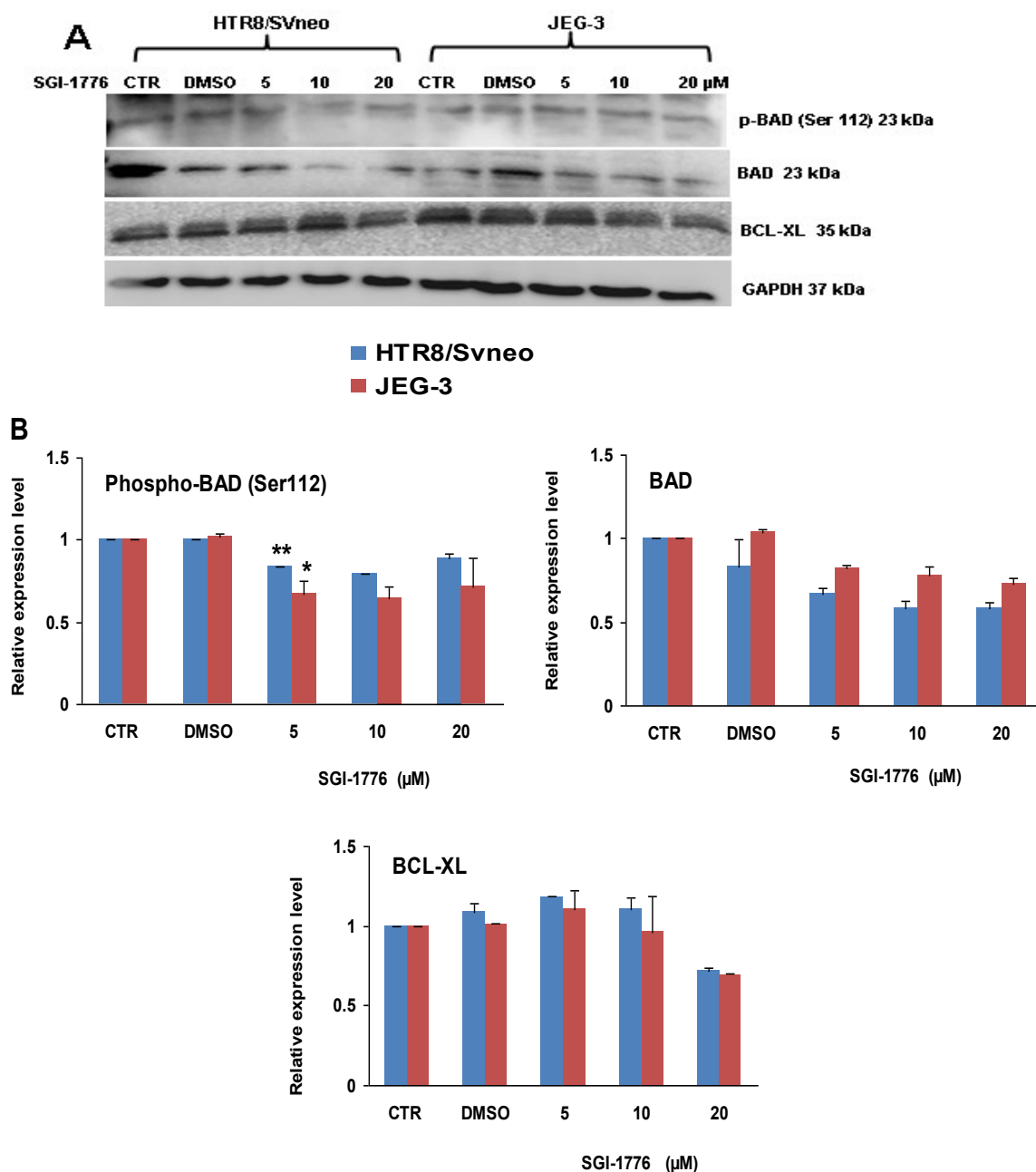
### 4.3.2 SGI-1776 effect on potential PIM Kinase targets

To elucidate the action mechanisms of PIM Kinases, we evaluated the effect of the inhibitor SGI-1776 on PIM kinase functions. HTR8/SVneo and JEG-3 cells were treated with vehicle DMSO (0.01%) alone or different concentrations of SGI-1776. Cells were harvested after 24 h incubation and compared with untreated cells. The total and phosphorylated protein expression of the PIM kinase target BAD were analyzed by immunoblot analysis (Fig 22. A).

The phosphorylation level of BAD (ser112) compared with total protein was measured (Fig 22.B). In HTR8/SVneo and JEG-3 cells, total BAD protein was found to have no change whereas Phosphorylated BAD was decreased at SGI-1776 concentrations of 5  $\mu$ M. in both cell lines. There was also no significant change in the expression of anti-apoptotic proteins BCL-XL (Fig 22.B).

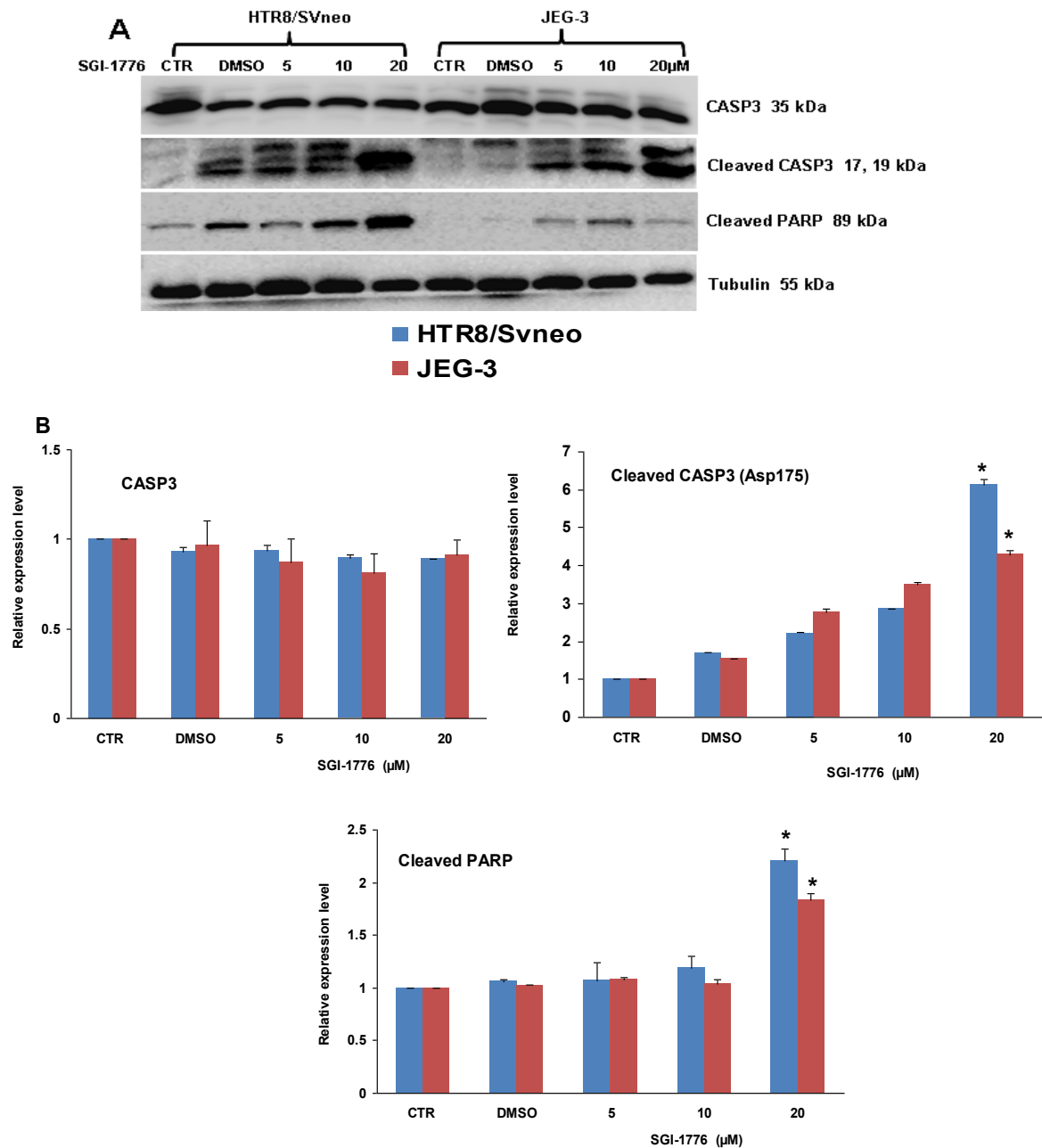
Apoptosis induction was confirmed by immunoblots for PARP and CASP 3 (Fig 23.A). By densitometry analysis of the blots, a 20 $\mu$ M SGI-1776 induced a significant increase of cleaved PARP and cleaved CASP 3 expression with no significant change in total CASP-3 expression in both cell lines (Fig.23.B).

In addition to BAD, another target of PIM Kinases phosphorylation is MYC. Overexpression of PIM Kinases are frequently associated with elevated MYC levels. Since MYC has an important role in tumor biology, we evaluated the effect of SGI-1776 on its phosphorylation in HTR8/SVneo and JEG-3 cells, phosphorylation of MYC at ser62 and total MYC by immunoblotting (Fig. 24. A).The densitometry analysis showed a significant decrease in the relative expression in c-MYC and p-C-MYC expression after treatment with SGI-1776 in a dose dependent manner (Fig.24.B)



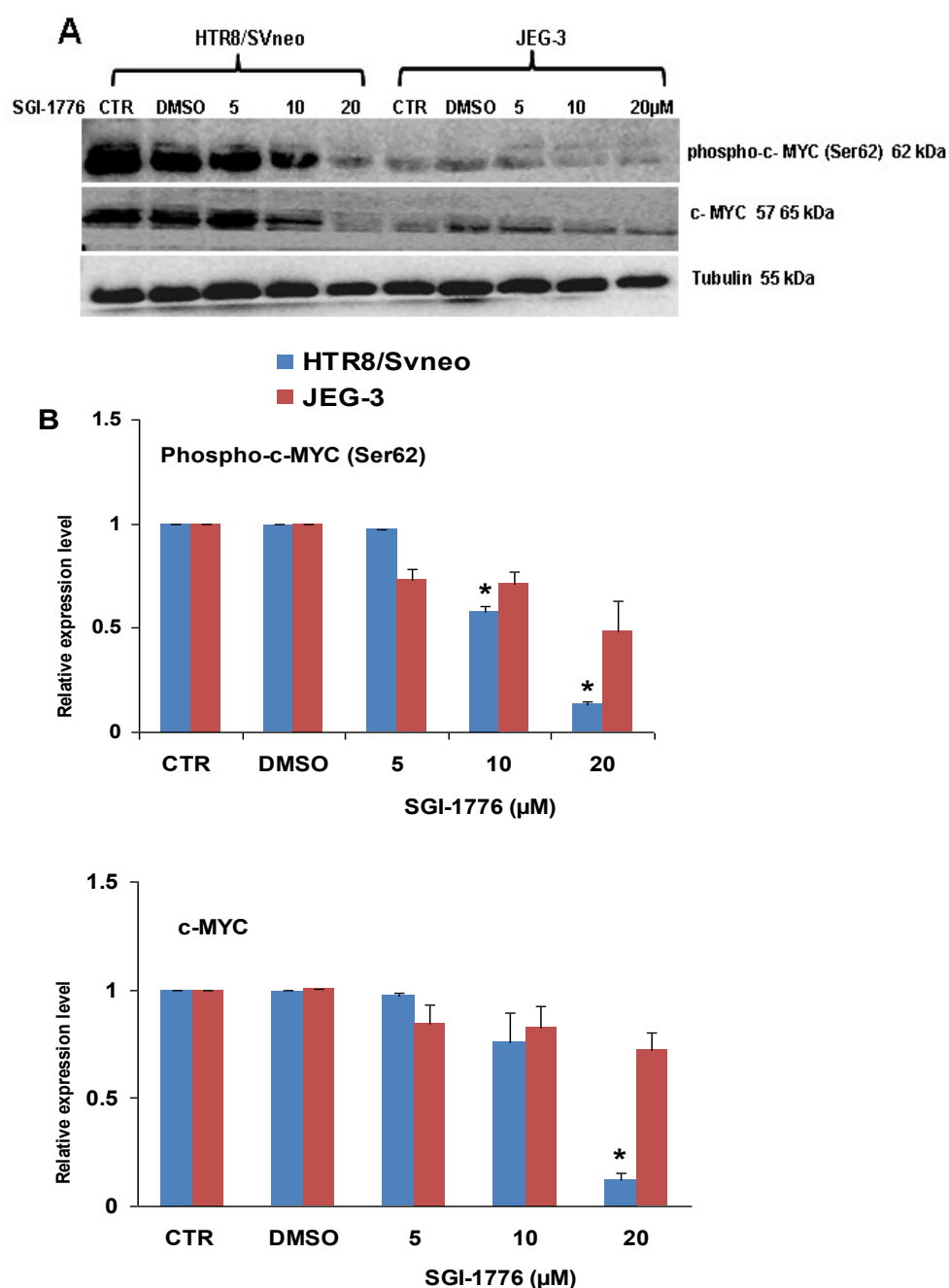
**Figure 22.** Immunoblot analysis of pro-apoptotic protein.

(A) Immunoblot analysis of potential target proteins in HTR8/Svneo and JEG-3 cells. Cells were treated with 0.1% DMSO alone or with 5, 10, or 20  $\mu$ M/ml SGI-1776 for 24 h. (B) (C) and (D) Densitometry analysis of potential PIM kinase target proteins in HTR8/Svneo and JEG-3 cells. The protein levels of p-BAD (ser112), total BAD and BCL-XL were normalized to GAPDH as loading control (n=3 independent experiments, mean  $\pm$  SD, \* p<0.05, \*\* p<0.01 Student's t-test).



**Figure 23.** Immunoblot analysis of anti-apoptotic proteins.

(A) Immunoblot analysis of apoptosis indicating proteins (cleaved PARP, CASP-3, cleaved CASP-3) in HTR8/SVneo and JEG-3 cells. Cells were treated with 0.1% DMSO alone or with 5, 10, or 20  $\mu$ M/ml SGI-1776 for 24 h. (B) Densitometry analysis of potential target proteins in HTR8/SVneo and JEG-3 cells. The protein levels of CASP3, cleaved CASP3 and cleaved PARP were normalized to tubulin loading control (n=3 independent experiments, mean  $\pm$  SD, \* p<0.05, Student's t-test).

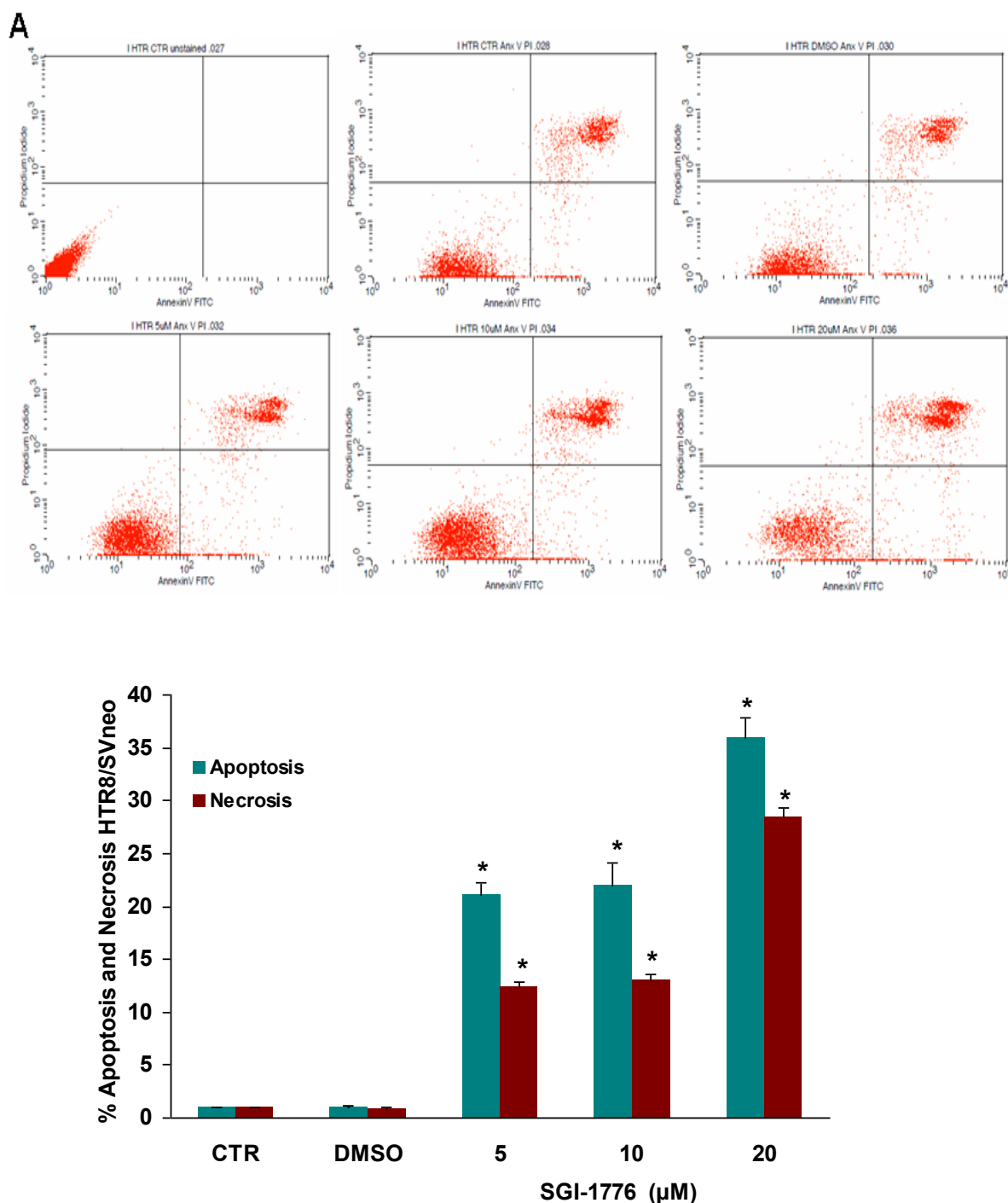


**Figure 24.** Protein analysis of Potential targets of PIM kinases

(A) Immunoblot analysis of potential PIM Kinase target protein c-MYC in HTR8/SVneo and JEG-3 cells. Cells were treated with 0.1% DMSO alone or with 5, 10, or 20  $\mu$ M/ml SGI-1776 for 24 h 10 (B) Densitometry analysis of p-c-MYC and c-MYC in HTR8/SVneo and JEG-3 cells. (n=3 independent experiments, mean  $\pm$  SD, \*  $p < 0.05$ , Student's t-test).

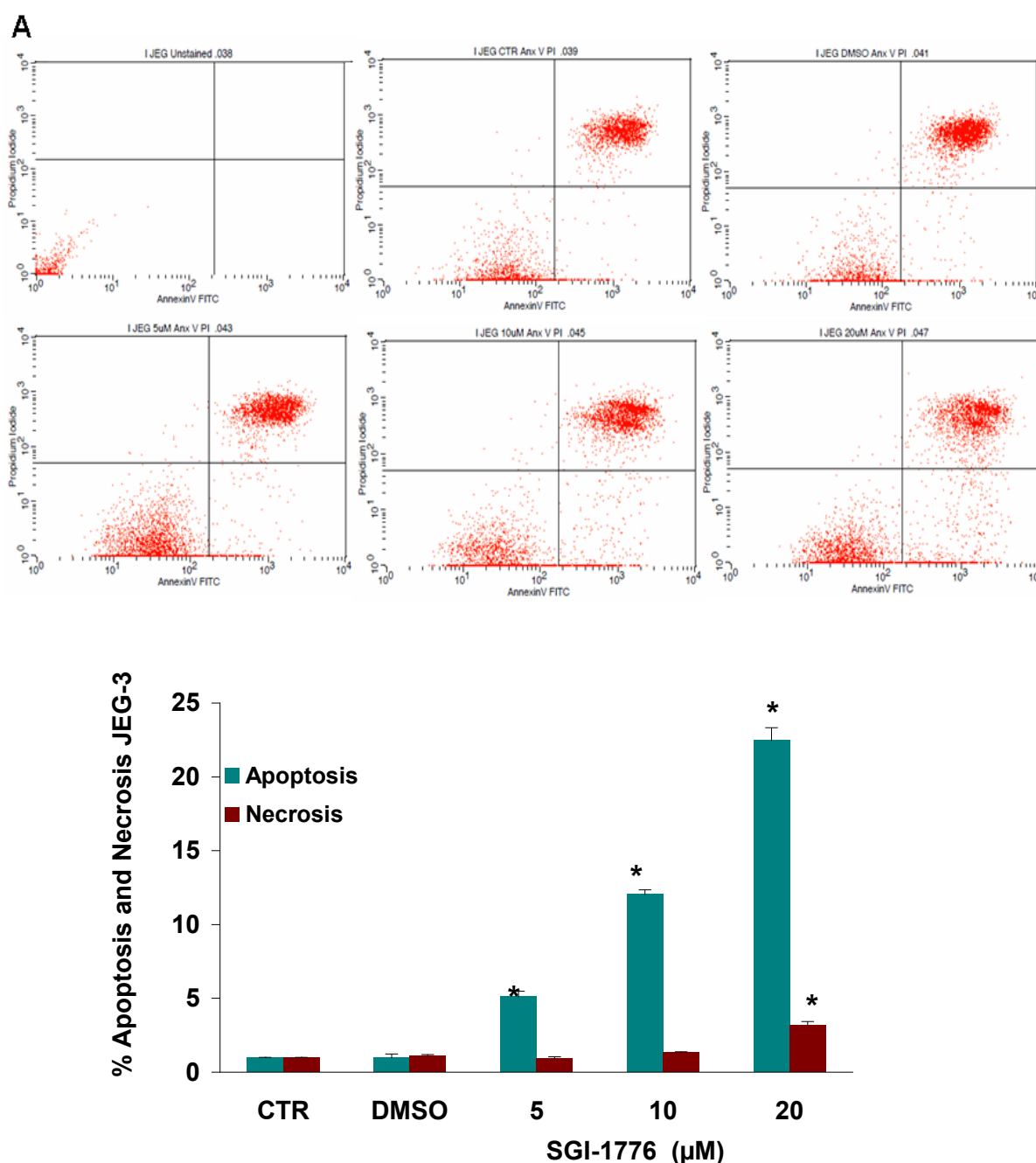
### 4.3.3 Apoptosis induction by SGI-1776

Both cell lines HTR8/SVneo and JEG-3 cells were treated with different concentrations of SGI-1776, and then stained with annexin V-FITC/PI and analyzed by flow cytometry to measure the apoptosis induction (Fig. 25 and Fig. 26.). HTR8/SVneo cells were incubated for 24 h with 5, 10 and 20  $\mu$ M/ml SGI-1776, which resulted in a significant increase in apoptosis (Fig. 25.A). After treating JEG-3 cells with 20  $\mu$ M SGI-1776 for 24 h, a significant increase in the apoptosis of more than 20% was observed (Fig. 26.A).



**Figure 25.** Apoptosis assay by annexin V showing the cell death in HTR8/SVneo cells.

(A) Flow cytometry for Apoptosis assay by annexin V showing the cell death in HTR8/SVneo cells treated with SGI-1776. Cells were either treated with 0.1% DMSO alone or treated with 5, 10, 20  $\mu\text{M}/\text{ml}$  SGI-1776 and cells were harvested after 24 h and stained with annexin V and Propidium Iodide (PI). Lower panel represents the relative percentage of Apoptosis in HTR8/SVneo cells respectively. (n=3- independent experiments, Mean  $\pm$  SD \*  $P < 0.05$ ).



**Figure 26.** Apoptosis assay by annexin V showing the cell death in JEG-3 cells

(A) Flow cytometry for Apoptosis assay by annexin V showing the cell death in JEG-3 cells treated with SGI-1776. Cells were either treated with 0.1% DMSO alone or treated with 5, 10, 20  $\mu$ M/ml SGI-1776 and cells were harvested after 24 h and stained with annexin V and Propidium iodide (PI). Lower panel represent the relative percentage of apoptosis in JEG-3 cells respectively. (n=3- independent experiments, Mean  $\pm$  SD \*  $P < 0.05$ ).

## **PART II**

### **4.4 HTR8/SVneo cell characterization of Stem cell property**

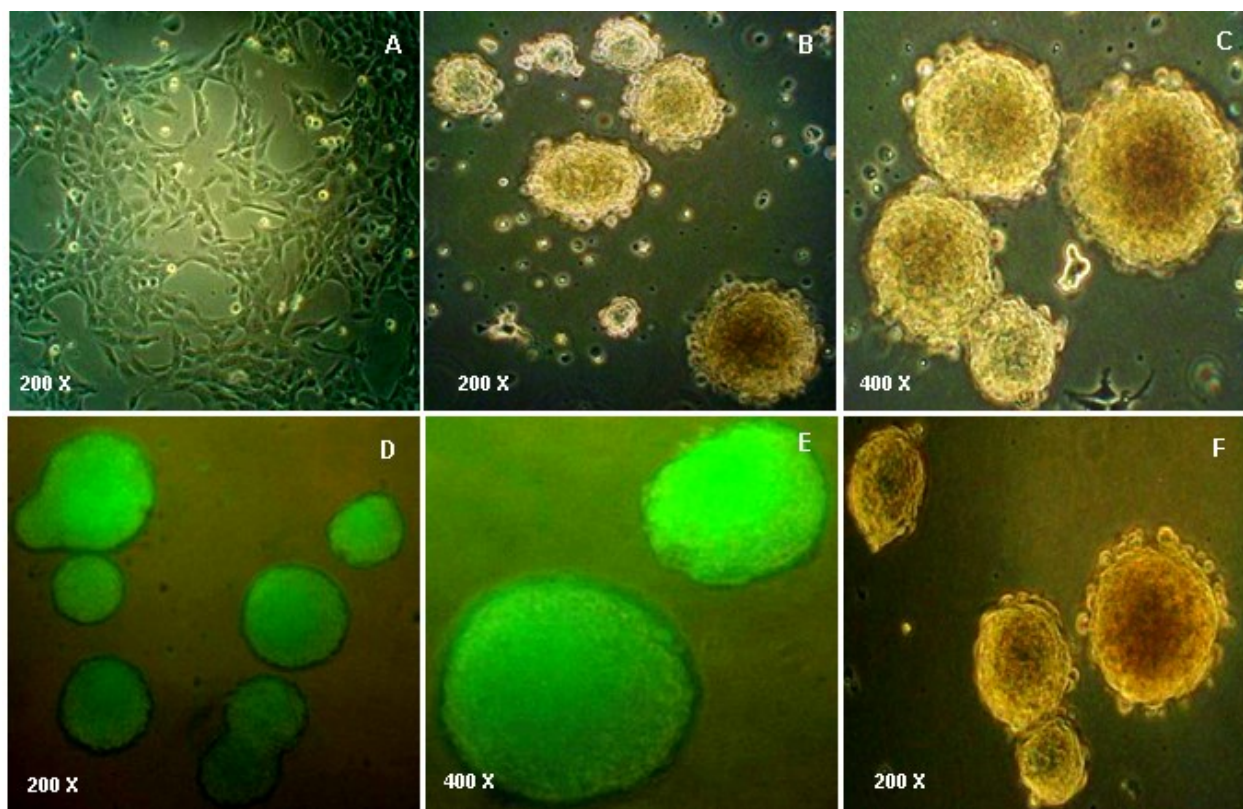
#### **4.4.1 Characterization of spheroids formed from HTR8/SVneo cells**

HTR8/SVneo cells were used as a model to investigate cancer stem cell like (CSC) properties exhibited upon 3-dimensional (3 D) culture by generating spheroids on a non-adherent surface.

#### **4.4.2 Morphology and cell viability by microscopic analysis**

Spheroid were generated seeding cells at a density of  $1 \times 10^6$  cells on petri dishes (non-adherent) with or without stimulation with LIF (10 and 50 ng/ml) and incubated for up to 72 h under standard conditions. Formed spheroids were further analyzed and compared with cells cultured in conventional 2D models (Fig.27.A-C). Spheroids are small round-shaped aggregates of cells with diameters of approximately 50-500 $\mu$ m. Spheroids were incubated with cell tracker fluorescence dye, which stains the viable cells (Fig.27.D-E) and Spheroids treated with LIF showed no change in morphology and number of spheroids generated (Fig. 27 F).



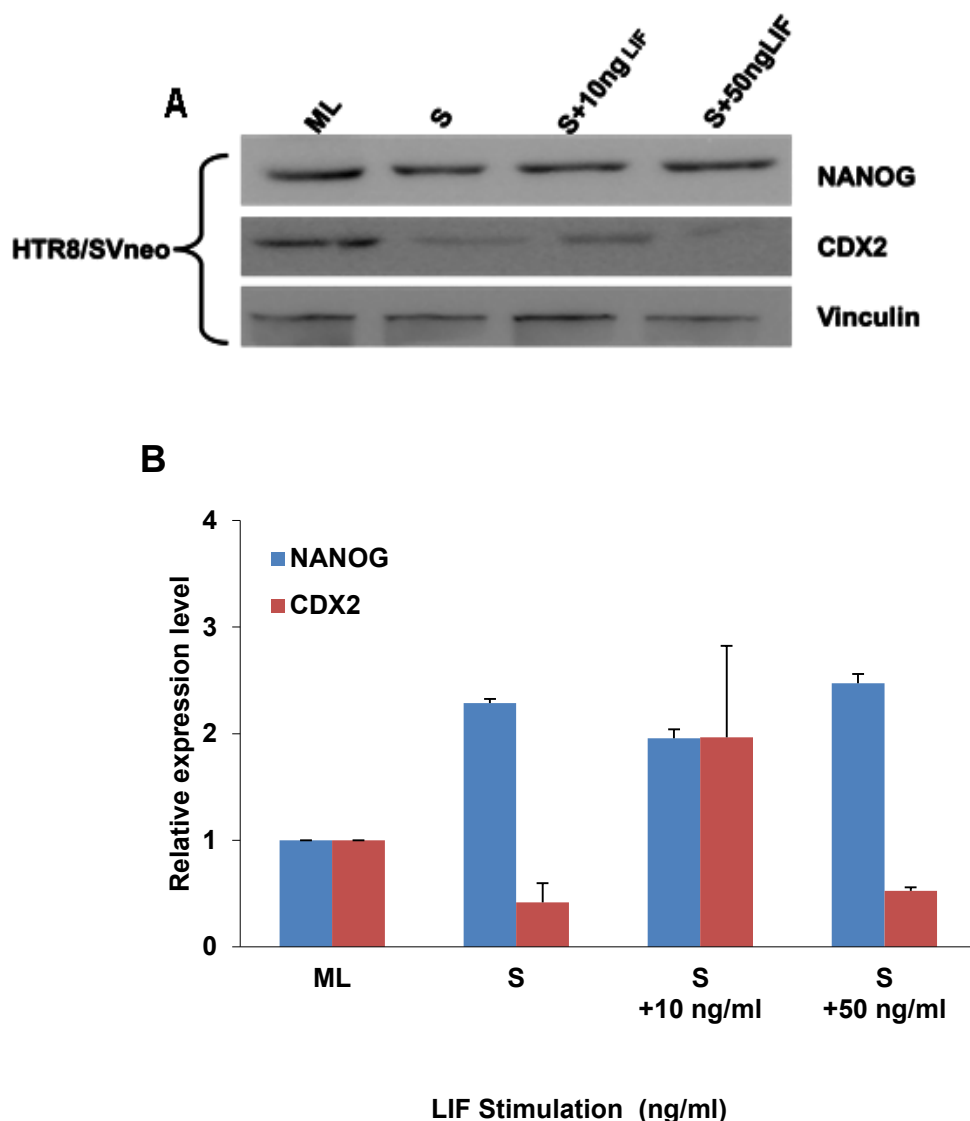


**Figure 27.** Spheroid formation in the HTR8/SVneo cell line

(A) Initial monolayer morphology 200x; (B) 200X and (C) 400X: spontaneous spheroid formation on day 3 on non-adherent surface at 200 $\mu$ m and 400 $\mu$ m. (D) and (E) Viability test of spheroids via cell tracker staining at 200X and 400X respectively; (F) spheroids after LIF stimulation (no change in morphology, however tendency to form lower number of spheroids).

#### 4.4.3 Western Blot analysis

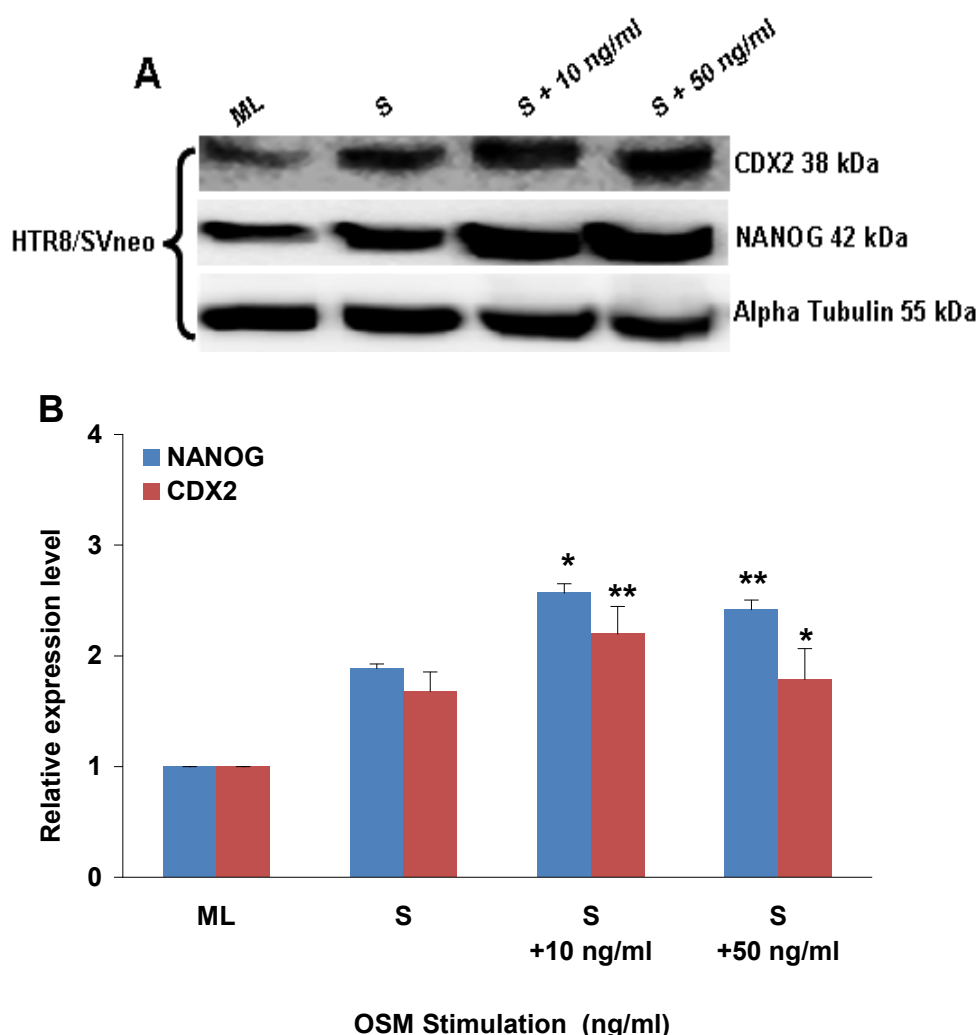
Western blot analysis was performed with spheroids generated for stem cell transcription factors NANOG, CDX2, SOX2 and OCT4A. We detected, that spheroids formed from HTR8/SVneo cells express transcription factors involved in maintenance of stemness. These include CDX2 which is the most specific trophoblast stem cell marker and NANOG. Upon LIF stimulation, the expression of transcription factors was altered. NANOG expression increased and CDX2 expression (Fig. 28.A) decreased significantly in spheroids with 50 ng/ml LIF stimulation compared with control monolayer cultures. Vinculin served as housekeeping reference. SOX2 and OCT4A were not expressed, (data not shown).



**Figure 28.** Transcription factor for stem cell upon LIF stimulation.

(A) Western blot detection of CDX2 and NANOG, (B) Densitometry analysis of stem cell-related transcription factors in HTR8/Svneo monolayers and spheroids formed with/out LIF stimulation. Vinculin was used as reference protein, experiments accomplished in three independent experiments.  $n=3$  independent experiments, mean  $\pm$  SD, \*  $p<0.05$ , Student's t-test). ML-Monolayer, S-Spheroid.

Upon OSM stimulation, the expression of transcription factors was altered. NANOG and CDX2 expression was increased (Fig. 28.A) significantly in spheroids with 50 ng/ml OSM stimulation compared with control monolayer cultures. Alpha tubulin served as housekeeping reference. SOX2 and OCT4A were not expressed, (data not shown).



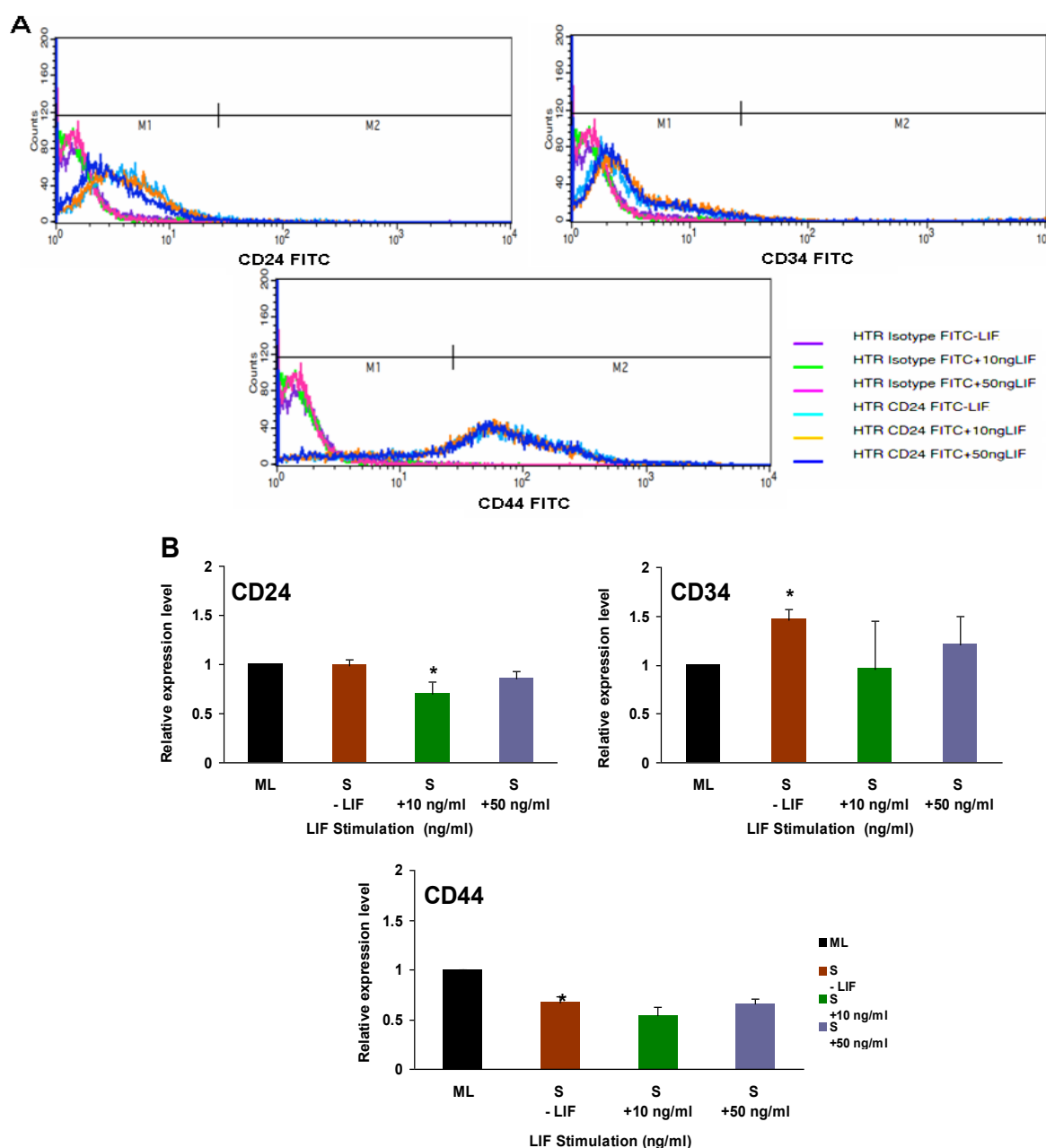
**Figure 29.** Transcription factor for stem cell upon OSM stimulation

(A) Representative Western blots for detection of CDX2 and NANOG in HTR8/SVneo cells grown in monolayers or spheroids stimulated with different concentrations of OSM, (B) densitometry analysis of respective Western blots. Alpha tubulin was used as reference protein, experiments accomplished in three independent experiments.. Error bars indicate standard deviation. ML-Monolayer, S-Spheroid.

#### **4.4.4 Flow cytometer analysis**

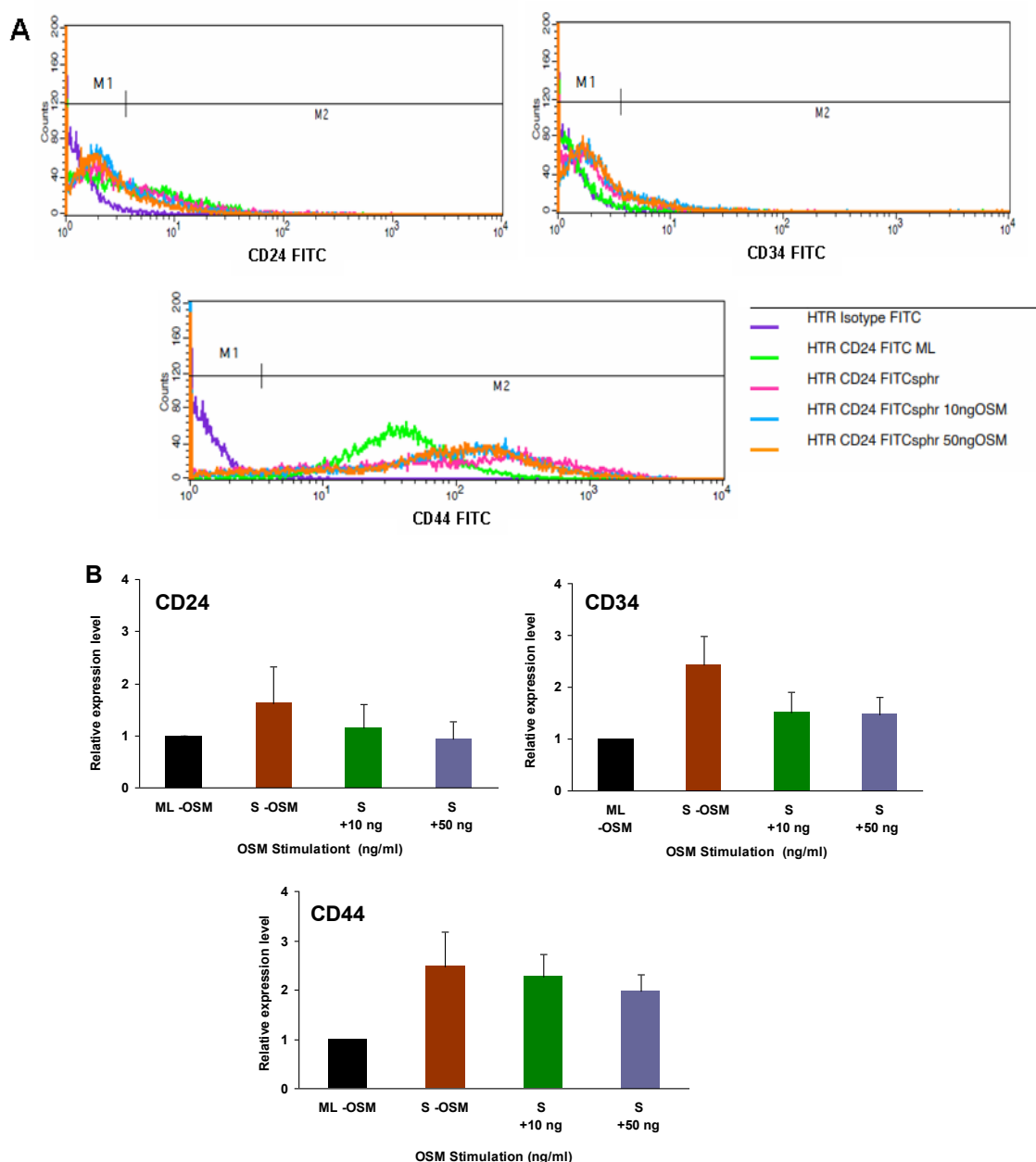
Flow cytometry analysis of HTR8/SVneo after LIF and OSM stimulation with different concentrations (10ng/ml and 50ng/ml) revealed alteration of surface molecules CD24, CD34 and CD44. In LIF treated spheroids cells there is significant decrease in the expression of CD24 at 10 ng/ml, upon LIF stimulation but rather the untreated spheroids showed significant increase in the expression of CD34 and CD44 showed interestingly low expression in spheroid but with no significant difference in LIF treatment (Fig 30. A).

In OSM treated cells there was no significant difference observed in the surface molecules by flow cytometry analysis (Fig 31. A).



**Figure 30.** Flow cytometry analysis for stem cell property upon LIF stimulation.

Upper panel (A) Histograms from flow cytometric analysis of stem cell surface marker expression on HTR8/SVneo spheroids formed with or without LIF stimulation for 72 h. M1 indicates negative cells as defined by application of isotype control antibodies; M2 indicates positive cells. Lower panel (B) Spheroids was generated from HTR8/SVneo cells stimulated with two different concentrations of LIF or without LIF. Expression of stemness-related surface markers was assessed by flow cytometry  $n=3$  independent experiments, mean  $\pm$  SD, \*  $p<0.05$ , Student's t-test). ML-Monolayer, S-Spheroid.



**Figure 31.** Flow cytometry analysis for stem cell property upon OSM stimulation.

Upper panel (A) Histograms from flow cytometric analysis of stemness-related cell surface marker expression on HTR8/SVneo monolayers (ML) and spheroids (S) formed with or without OSM stimulation for 72 h. M1 indicates negative cells as defined by application of isotype control antibodies; M2 indicates positive cells. Lower panel (B) Quantification of flow cytometry results. Expression of stemness-related surface molecules in monolayers was defined as 1 and that in spheroids was calculated, respectively (n=3; error bars indicate standard deviation).

## CHAPTER 5 DISCUSSION

PIM kinase was reported first in late 1980s in murine leukemia virus (MuLV) that was related to transcriptional activation of Pim-1 gene with enhanced expression (Cuypers et al. 1984) (Selten et al. 1984). So far, its expression has been reported in almost all kind of human cancer (marking its presence to be important in cell survival, cell progression, cell migration, cell proliferation and apoptosis (Breuer et al. 1989). There is evidence that its overexpression leads to complications and is responsible for tumor progression. PIM kinases have three isoforms and all of them are very similar to each other which may lead to functional interference impairing growth and survival of cancer (Cuypers et al. 1984). Aberrant expression of PIM kinases is observed in pancreatic cancer and plays pivotal roles in the regulation of cell cycle, apoptosis, and properties of stem cells, metabolism, autophagy, drug resistance and targeted therapy. Blocking the activities of PIM kinases could prevent pancreatic cancer development (Xu et al. 2014b). PIM kinases are not only involved in cancer progression but it has been demonstrated that PIM kinases play an important role during embryonic development (Aksoy et al. 2007, Eichmann et al. 2000). These findings opened the path to study the role of PIM kinases during pregnancy, especially on trophoblast cell functions. The role of PIM kinase in trophoblast cells is unknown so far. The present study analyzes their expression and potential involvement in LIF-induced intracellular signaling. The current study demonstrates that LIF increases PIM kinase protein expression in trophoblastic cell lines. The increase in PIM1 and PIM3 in HTR8/SVneo cells upon LIF stimulation appeared to be consistent with the finding by another group (Aksoy et al. 2007) where PIM1 and PIM3 was unregulated by LIF via activation of the STAT3 pathway.

PIM1 expression can be induced by several external stimuli in particularly by cytokines relevant in the immune system, which led to coining PIM1 kinase as a “booster” for the immune response (Bachmann und Moroy 2005). There are no comparisons so far related to PIM kinase functions between human trophoblast choriocarcinoma. Hence, in this study we have analyzed and compared the mRNA expression of PIM kinases in normal primary term trophoblast cells, the immortalized cell line HTR8/SVneo and the choriocarcinoma cell line JEG-3.

The results obtained from this study provide information about the expression of all three isoforms. PIM1 and PIM3 is evidently increased upon LIF stimulation in trophoblast cells, similar to the finding in inducing endothelial differentiation of cardiac stem cell via PIM1 up regulation (Iwakura et al. 2011). PIM3 was found to be highly enhanced in pre-malignant tissues in human hepatocellular carcinoma (Fujii et al. 2005).

PIM1 and PIM2 have been reported to be highly expressed in murine cells of hematopoietic origin and PIM kinases regulate the stability of SOCS-1 (Chen et al. 2002). SOCS have been found to be regulated by cytokines in trophoblast cells (Fitzgerald et al. 2009). STAT3 mediates trophoblast invasion by LIF induction and are involved in the fine regulation of immunological and cellular responses (Fitzgerald et al. 2009) (Poehlmann et al. 2005b). PIM1 kinase is expressed in human myeloid cells, which was induced by IL-3, G-CSF, GM-CSF and IL-6, which is supporting our study, results where LIF is a pleotropic function cytokine belonging to the IL-6 family (Lilly et al. 1992). This cytokine family is responsible for many biological responses that include immune response, inflammation, hematopoiesis and tumorigenesis by regulating the cell growth, survival and differentiation. All members use the common subunit receptor gp130 which activates the JAK/STAT pathway, where STAT3 plays a vital role in transmitting the signal from the cell membrane to the nucleus (Hirano et al. 2000). PIM1 kinase is associated esophageal squamous cell carcinoma. Its overexpression is related with poor prognosis (Liu et al. 2010). PIM1 promotes tumorigenicity in human prostate cancer cell lines by c-MYC driven transcription activity (Kim et al. 2010). PIM1 protein expression is regulated by its 5'-untranslated region and Eukaryotic translation initiation factor 4E (eIF-4E) (Hoover et al. 1997). PIM1 mRNA expression in prostate cancer serves as a diagnostic marker (He et al. 2007). Due to high frequency of PIM1 expression in head and neck squamous cell cancer of different grades and stages in conjunction with its absence in non-neoplastic head and neck squamous cell epithelium underlines the functional role of PIM1 in malign molecular processes (Beier et al. 2007). Frequent down-regulation of PIM1 mRNA expression in non-small cell lung cancer has been found in association with lymph node metastases (Warnecke-Eberz et al. 2008). PIM1 is a direct transcriptional target of HOXA9 and a mediator of its anti-apoptotic and pro-proliferative effects in early cells.



HOXA9 is frequently up-regulated in acute myeloid leukemia (Hu et al. 2007). It is also involved in endometrial receptivity which suggests a connection with PIM1 in early pregnancy (Xu et al. 2014a). PIM2 kinase plays a key role in ovarian cancer cells (Musiani et al. 2014). Survival under hypoxic conditions in cancer cells takes place as they remodel glucose metabolism to support tumor progression. HIF transcription factor is essential for cellular response to hypoxia. A regulatory feedback loop between HIF-1 $\alpha$  and PIM2 in hepatocellular carcinoma (HepG2) cells was observed where PIM2 expression was induced upon hypoxia in a HIF-1 $\alpha$ -mediated manner. HIF-1 $\alpha$  induced PIM2 expression, a regulatory mode which may be important for the adaptive responses and antagonizing hypoxia during tumor progression (Yu et al. 2014). Compared to benign, inflammatory and pre-malignant conditions, expression of PIM1 mRNA and protein increased significantly in pancreatic malignancies and the hypoxic cells displayed higher PIM1 mRNA and protein levels. So, the presence of PIM1 in tumor has a positive prognostic impact (Reiser-Erkan et al. 2008). PIM3 kinase is expressed in endothelial cells, promoting vascular tube formation (Zhang et al. 2009) (Yang et al. 2011). The work presented here demonstrates that all three isoforms of PIM kinases are constitutively expressed in trophoblast cells. Upon LIF stimulation the expression increases in both cell lines which are chosen for this study. PIM1 kinase bound to Hsp70 or Hsp90 remains active, emphasizing the importance of its overall cellular levels. PIM1 levels can be modulated in cells through degradation and stabilization. On protein level, the stability of PIM1 is regulated by Hsp90, Hsp70, and the ubiquitin-proteasome pathway (Shay et al. 2005). Upon LIF stimulation PIM1 expression in our trophoblast and choriocarcinoma cells was found to be localized in nuclei and cytoplasm as assessed by immunocytochemistry (ICC) and immunofluorescence (IF) staining. These observations coincide with findings of PIM1 expression in nuclei in normal gastric epithelial cells, but more pronounced in cytoplasm of human gastric tumors (Yan et al. 2012). PIM1 kinase expression in adipocytic neoplasms was localized to the cytoplasm (Nga et al. 2010) (Telerman et al. 1988). These findings are consistent with our current findings where immunostaining showed the expression of PIM1, PIM2 and PIM3 in both HTR8/SVneo and JEG-3 cell lines. PIM1 and PIM3 expression was higher than PIM2 expression in HTR8/SVneo cells whereas in JEG-3 cells, PIM2 and PIM3 were more expressed than PIM1.

Our investigation could demonstrate that PIM kinases play role in trophoblast cells during pregnancy. We have proven that LIF regulates this molecule which makes us to interpret, that LIF is the central mediator of both PIM induction and JAK/STAT pathway activation.

In our study, the inhibition of PIM kinases resulted in induction of apoptosis and decrease in cell viability and cell proliferation. PIM kinase signaling is involved in many pathways, and thus the biological effects of its inhibitor SGI-1776 in trophoblast in comparison to choriocarcinoma cells might be multifaceted. SGI-1776 is a specific inhibitor for all three isoforms of PIM kinase activity. Significant transcription inhibition was observed in an acute myeloid leukemia (AML) cell line and in primary cells, which is similar to our findings that c-MYC driven transcription is disrupted by PIM kinase inhibition due to the decrease c-MYC (Ser62) phosphorylation (Fig 11.A) which is needed for c-MYC stability (Chen et al. 2011) (Kim et al. 2013). SGI-1776 treatment in 4 Mantel cell lymphoma (MCL) cell lines resulted in apoptosis induction and Phosphorylation of transcription (c-MYC) (Yang et al. 2012). On cell viability nad proliferation extensive biochemical characterization of SGI-1776 confirmed its specificity for the three isoforms of the PIM family. Treatment of prostate cancer cells with SGI-1776 resulted in a dose-dependent reduction in phosphorylation of PIM kinase substrates that are involved in cell cycle progression and apoptosis. SGI-1776 was able to reduce cell viability in a multidrug resistant prostate cancer cell line (Mumenthaler et al. 2009). Inhibition of PIM3 kinase causes growth inhibition of cancer cells by down regulating the expression of pSTAT3 (tyr705) (Chang et al. 2010). Targeting PIM2 kinase by biochemical inhibitors impaired cell growth, decreased cisplatin-triggered BAD phosphorylation, and sensitized ovarian cancer cells to drug-induced apoptosis (Musiani et al. 2014). PIM2 kinase is an important target of treatment for tumor progression and bone loss in myeloma (Hiasa et al. 2015). Chronic lymphocytic leukemia (CLL) cells when treated with SGI-1776 resulted in a dose-dependent induction of apoptosis. Levels of the ant apoptotic protein BCL-XL were unchanged. However, levels of total c-MYC as well as phospho-c-MYC (Ser62), a PIM1 target, were decreased after SGI-1776 treatment (Chen et al. 2009b).

In murine hematopoietic cells PIM2 kinase phosphorylates BAD (ser112) and prevents BAD-induced cell death (Yan et al. 2003). PIM1 kinase promotes inactivation of the pro-apoptotic BAD protein by phosphorylating it at the ser112 gatekeeper site (Aho et al. 2004). The *c-myc* gene is induced in response to the proliferative signals elicited by extracellular stimuli, including IL-6. The non-receptor tyrosine kinases c-src (Barone und Courtneidge 1995) and PIM1 induce BCL-2 expression and inhibits c-MYC-induced apoptosis in a manner that is dependent on gp130-induced signals. PIM1 kinase acts as activator of the cell cycle pathway in neuronal death induced by DNA damage (Zhang et al. 2010).

Further, stem cell-like features in three-dimensional (3D) in vitro cultures are used in cancer research as intermediate models between in vitro cell line cultures and in vivo tumor models. Spheroid (3D) cancer cell models have gained popularity in stem cell research. Spheroids and spheroid-like structures are characterized by their well-rounded shape, the presence of cancer stem cells, and their capacity to be maintained as free-floating cultures. A multicellular tumor spheroid model was first described in the early 70s and obtained by culture of cancer cell lines under non-adherent conditions (Weiswald et al. 2015). In addition, we describe here applications of spheroid cultures in trophoblast research to investigate the potential role of LIF which not only supports self-renewal of ESCs but also has a function in extra-embryonic development, where it promotes trophoblast proliferation, differentiation, and invasion (Poehlmann et al. 2005a) (Prakash et al. 2011) (Takahashi et al. 2003). Upon LIF withdrawal, ESC differentiate but not to neural lineages (Ying et al. 2003). LIF supported the expansion of ESC but did not induce NANOG expression. LIF supports a totipotent state comparable to early embryonic cells that co-express embryonic and extra-embryonic determinants (Morgani et al. 2013). There are reports suggesting suitable surface markers to characterize the stem cell-like features, which are CD44, CD34, CD24 and CD133.

CD44, a hyaluronic acid receptor, is one of the most commonly studied surface markers, which is expressed by almost every tumor cell. CD24, a heat stable antigen, is another surface marker expressed in many tumor types (Jaggupilli und Elkord 2012) (Ghosh et al. 2012). From the adenocarcinoma cell line generated spheroids express CD44 and CD133 as analyzed by flow cytometry (Liu et al. 2015).

In our study, we have characterized stem cell-like properties in HTR8/SVneo trophoblast cell models, by analyzing the surface molecules CD44, 34, 24 and CD133, and the transcription factors NANOG and CDX2. STAT3 signaling regulates embryonic stem cell fate in a dose-dependent manner (Tai et al. 2014). CD44 and CD24 were used as CSC markers alone or in combination to identify cancer stem cells in tumors of the human female reproductive system (Lopez et al. 2013). Tumorigenic pancreatic cancer cells have been identified using the cell surface antigens CD44, CD24, and CD133 (Rasheed und Matsui 2012). Human primary colorectal cancer expresses specific phenotypic profiles, including CD133, CD24, and CD44 (Muraro et al. 2012) (Wang et al. 2012). Liver CSCs can be identified by several cell surface antigens, including CD133, CD90, CD44, Epithelial cell adhesion molecule (EpCAM) and CD133 (Liu et al. 2011) Breast cancer stem cells express the cell surface phenotype CD44+CD24-/low (Ginestier et al. 2007). High levels of CD44 expression in breast cancer can be used as stem cell marker (Gong et al. 2015). In breast cancer stem cells, CD44 is involved in regulation of Notch 4 receptor signaling (Harrison et al. 2010). CD44 positive cells are characteristic markers for mesenchymal stem cells (NguyenThai et al. 2015). CD44 is expressed in hepatocellular carcinoma (Feng et al. 2014) and in lung cancer (Alamgeer et al. 2013). A highly tumorigenic subpopulation of pancreatic cancer cells expresses the cell surface markers CD44, CD24, and epithelial-specific antigen (ESA) (Li et al. 2007). Also head and neck squamous cell carcinomas express CD44 surface antigen (Sterz et al. 2010).

Non-small cell lung cancer cells expressing CD44 have increased stem cell-like features (Leung et al. 2010). Therapeutic targeting of stem-like cancer cells via STAT3 modulation using a nanomedicine approach selectively reduces the CD44+ stem cell population in melanoma (Misra et al. 2015). CD34 is expressed in peripheral blood stem cells. It functions as adhesion molecule important for cell migration and regulation in hematopoiesis (Dercksen et al. 1995). Trans membrane CD34 glycoprotein is the most important marker for identification, isolation and enumeration of hematopoietic stem cells (HSCs) (Shafaghat et al. 2015). Hence the above mentioned studies, supports our finding of HTR8/SVneo cells expressing CD44, CD34 and CD24, which might be potential surface markers for characterizing stem cell-like features.

Transcription factors responsible for stem cells are many out of which we analysed the expression of NANOG and CDX2, these factors in human pluripotent stem cells is found to be highly expressed in colonies spontaneously differentiated in multiple lineages, including trophoblast cell lines. Cell lines exhibit totipotent potential and these properties may have implications for regulation of lineage decisions in the early embryo (Yang et al. 2015). NANOG stabilizes embryonic stem cells in culture by resisting or reversing alternative gene expression states (Chambers et al. 2007). Overexpression of NANOG reduces heterogeneity during embryonic stem cell maintenance. Interestingly, NANOG heterogeneity does not correlate with the heterogeneous expression of stage-specific embryonic antigen-1, suggesting that multiple but overlapping levels of heterogeneity may exist in embryonic stem cells (Singh et al. 2007). FACS analyses show that multipotent cytotrophoblast cells from human first trimester chorionic villi cells express markers typical for human stem cells such as SSEA4, OCT-4, ALP, and CD117. These are present within the cytotrophoblastic tissue of human fetal chorionic villus samples (CVSs) as also OCT-4, NANOG, and SOX2 transcripts (Spitalieri et al. 2009). The differentiation of human embryonic stem cells down regulate the trophoblast lineage, the stemness markers POU5F1 (OCT4) and NANOG were markedly down-regulated, followed temporally by up-regulation of the CDX2, KRT7, HLA-G, ID2, CGA, and CGB trophoblast markers in presence of BMP4 (Marchand et al. 2011). Isolated and re-aggregated trophectoderm (TE) cells from full human blastocysts are able to develop into blastocysts with inner cell mass (ICM) cells expressing the pluripotency marker NANOG. In our hands, HTR8/SVneo cells expressed NANOG in monolayer culture and spheroids. Upon LIF stimulation, expression of NANOG at higher concentration (50 ng/ml) in spheroid culture was increased, whereas CDX2 expression was decreased at higher concentration, this might be due to the properties of LIF, as it is involved in feedback mechanism

Moreover, the majority of the isolated TE cells which were repositioned in the center of the embryo do not sort back to their original position but integrate within the ICM and start to express NANOG (De Paepe et al. 2013). Human trophoblast stem cells express gene markers of both the trophectoderm and the inner cell mass. They exhibited genetic and biological characteristics similar to those of human embryonic stem cells, yet genetically distinct from placenta-derived mesenchymal stem cells.

Overexpression of transcription factor NANOG was possibly achieved through a retinoic acid (RA)-induced non-genomic c-Src/Stat3/Nanog signaling pathway mediated by the subcellular c-Src mRNA localization for the maintenance of pluripotency in trophoblast neural stem cells (tNSCs) in rats (Lee et al. 2012). Developmental expression of a number of genes was similar to that in murine embryos (OCT3B/4, CDX2, and NANOG). However, GATA6 is expressed throughout pre-implantation development in the human when cultured in Heparin-binding epidermal growth factor (HBEGF). LIF appears to facilitate human embryo expression of a number of genes. CDX2 expression is regulated by LIF, removal of LIF, stimulated CDX2 expression and induced formation of trophoblast stem cells (TSC) which then were able to differentiate into cells spongiotrophoblast and trophoblast giant cells (He et al. 2008). This result is consistent to our finding where CDX2 expression is decreased upon addition of LIF. When MBD3 knockdown cells were cultured in ES medium without LIF or in trophoblast stem (TS) cell medium, these cells differentiate into TE cells, which express CDX2 in mouse ES cells (Zhu et al. 2009) (Suwinska et al. 2010). Trophoblast stem cells under defined culture conditions express CDX2 in mice (Ohinata und Tsukiyama 2014). Cell growth was accelerated in an undifferentiated state sustained by LIF in ES cells (Iha et al. 2012). CD44 interaction activates stem cell marker NANOG, STAT3-mediated multi drug resistant gene (MDR1) gene expression in breast and ovarian tumor cells (Bourguignon et al. 2008). NANOG and STAT3 signaling promote microRNA-21 expression and chemoresistance in hyaluronan/CD44-activated head and neck squamous cell carcinoma cells (Bourguignon et al. 2012).

Our model showed an increase expression of NANOG and CDX2 in the spheroids with OSM treatment, whereas there was no change in surface markers CD44, CD34 and CD24. OSM shares structure similarities and receptor with LIF (Rose und Bruce 1991). It has been reported in one of the study that OSM and LIF reveal some similar biological functions in trophoblastic cells (Chaiwangyen et al. 2014). Our studies exhibit that OSM and LIF (partially) induces the stem cell-like properties in trophoblast cell model HTR8/SVneo. To our knowledge, this is the first report that human OSM play a key role in maintaining stem cell-like properties.

## CHAPTER 6 CONCLUSION

In conclusion, there have been studies done on investigating many molecules that are involved in several ways contributing its role in regulating to achieve a successful pregnancy. LIF being very important molecule during pregnancy was taken in the current study to elucidate the functions of PIM kinases in trophoblast. We characterized the expression of PIM kinases upon LIF stimulation and inhibiting their activities by using PIM-Kinase inhibitor IX, SGI-1776 (chemical inhibitor). It was found that all the three PIM kinases (PIM1, PIM2 and PIM3) were expressed in both cell lines. The gene and protein expression level were shown to be increased upon LIF stimulation. Immunostaining data showed the localization and distribution of PIM kinases in both cells lines, respectively. The functional role of PIM kinase was elucidated in trophoblast cells where they play a vital role in cell survival and apoptosis. Further the trophoblast cell model has been characterized for stem cell like property by staining with surface antigen for studying the phenotype expression of the markers responsible for stem cell which are CD44, CD34, CD24 and CD133. The result demonstrated that cells were CD44+ve, CD34+ve, CD24+ and CD133-ve, proving the self-renewal property of HTR8/SVneo cell, and also expressed stem cell transcription factors NANOG, CDX2. These expressions were well studied as they were compared with monolayer culture 2D culture with 3D spheroid culture system, which provided the information regarding the cells ability to form spheroid that resembles stem cell-like features. Adding further, upon LIF stimulation, current study shows the decreased expression of surface markers, increased transcription factors at higher concentration, fulfilling another function of LIF to be having negative feedback loop mechanism. Hence these all above data provides a better understanding of the role of PIM kinases and stem cell-like features in trophoblast cells for successful pregnancy.

### ***Future Investigations***

To elucidate the PIM kinases expression in pregnancy pathology tissue samples and studies the functional analysis like migration, invasion in trophoblast cell models.

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“For I know the plans I have for you plan to prosper you and not to harm you, plans to give you hope and a future.” Jeremiah 29:11

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This PhD journey would be incomplete without my beloved parents, beautiful sisters without their eternal prayers, love, support and sacrifices. I would not have made it this far without them. I want to thank all the people from church for the love and prayers. Finally, I would like to thank God for creating me, wisdom, knowledge, strength to complete this work successfully and without you, everything is impossible. You always be with me.



# CURRICULUM VITAE



## Personal Information

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Date of Birth: 12.04.1985

Place of Birth: Port Blair, Andaman and Nicobar Islands, India

Nationality: Indian  
Resident in Germany

Marital Status: Single

## EDUCATION AND QUALIFICATIONS

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- 10/ 2011- present**      **Ph.D.** (Molecular medicine), university hospital  
Friedrich Schiller University, Jena, Germany.  
Ph.D. thesis: “Characterization of PIM kinase and  
cancer stem cell property in trophoblast
- 02/ 2011- 05/ 2011**      Boehringer Ingelheim Fonds(BI Fonds) –Travel  
Grant Travel  
Placenta Lab University hospital Jena Germany.
- 09/ 2009- 10/ 2010**      **M.Res. Cancer Biology (Biomedical Science),**  
Nottingham Trent University, Nottingham, UK.  
Master Thesis: “Invasive potentials of transformed  
placental cells and gestational trophoblastic tumor cells  
–A comparative analysis of placental versus tumor  
invasion”.  
Rank: First Class with Commendation (70%)
- 07/ 2006- 07/ 2008**      **M.Sc. Medical Laboratory Technology (MLT)**  
Loyola College, University of Madras, India  
Master Thesis: “Isolation and identification of  
different fungi causing keratitis-An Eye Opener”.  
Rank: First Class with Distinction College II Rank (78%)
- 07/ 2003- 05/ 2006**      **B.Sc. Microbiology,**  
Prof. Dhanapalan College for Women,  
University of Madras, India.  
Rank: First Class. (75%)
- 07/ 2001- 05/ 2003**      **High School** -Govt. Model Senior Secondary School,  
Port Blair, Andaman and Nicobar Islands, India.  
Rank: First class (70%)

## AWARDS AND ACHIEVEMENTS

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<b>04/ 2015-09/ 2015</b>	Short term grant DAAD University of Jena
<b>10/ 2011 -03/ 2015</b>	PhD Scholarship SIEMENS DAAD
<b>03/ 2011-05/ 2011</b>	Travel Grant Travel-Boehringer IngelheimFonds (BI fonds)
<b>09/ 2009-10/ 2010</b>	Scholarship for Bioscience, Nottingham Trent University.

## INTERNSHIP (work experience)

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<b>11/ 2010-02/ 2011</b>	<p>Practical Training Internship John van Geest Cancer Research Centre Nottingham Trent University. Topic:” Characterization of sub population of prostate cancer cell lines.</p>
<b>04/ 2008- 05/ 2008</b>	<p>Participated in the Quality Control Program Loyola, college. Chennai, India. Topic: Microscopy techniques: Sputum Analysis form tuberculosis</p>
<b>02/ 2008- 03/ 2008</b>	<p>Volunteer trainee lab technician (clinical laboratory) Loyola Health Centre, Chennai, India.</p>
<b>08/ 2007- 12/ 2007</b>	<p>Student assistant Department of Advanced Zoology and Biotechnology, School of Genomics, Loyola College Chennai, India. Topic: Trained on molecular Technique.</p>
<b>04/ 2007-05/ 2007</b>	<p>Summer Practical training Madras diabetic Research foundation, Chennai, India Topic: Biomedical instrumentation and molecular techniques.</p>

## TECHNICAL EXPERIENCE

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*Molecular Biology*: Genomic DNA and RNA isolation; sub cloning; generation; qPCR, MicroRNA transfection and analysis. Designing and standardizing primers.

*Immunology*: Antisera generation and monoclonal antibody isolation; isotopic, fluorescent, and biotin labeling of cellular proteins and antibodies; immunoprecipitation; radioimmunoassay; ELISA; FACS analysis; Immunostaining ; IHC; ICC; IF; TUNEL assay; Western blotting and troubleshooting.

*Biochemistry*: Spectrophotometric enzyme assays; protein quantitation; SDS PAGE.

*Microscopic techniques*: fluorescence microscopy; Laser scanning microscopy (work with software package)

*Tissue Culture*: Isolation of primary cells; mesenchyme stem cell, spheroid generation (3D culture) with different techniques, Micro vesicles (exosomes, micro particles) Mammalian adherent and non-adherent cell culture (2D culture); cell separation, including Percoll density gradient, microbiological techniques.

## CONGRESS AND MEETINGS

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- |                 |   |
|-----------------|---|
| <b>03/2015</b>  | International congress on embryo implantation and pregnancy.<br>New Delhi, India, Poster presentation.                                      |
| <b>09/ 2014</b> | International federation of Placenta Association (IFPA)<br>Congress Paris, France<br>Poster presentation.                                   |
| <b>04/ 2014</b> | 11 <sup>th</sup> Congress of the European Society for<br>Reproductive Immunology (ESRI), Poster presentation                                |
| <b>03.2014</b>  | International training in reproductive sciences and<br>Technologies, 4 <sup>th</sup> Jena InTreST-DGRM, Jena, Germany.<br>Oral presentation |
| <b>10/ 2013</b> | European Society for Reproductive Immunology (ESRI),<br>Kos Island, Greece.<br>Summer school participation.                                 |
| <b>09/ 2013</b> | International Federation of Placenta Association (IFPA)<br>Whistler British Columbia, Canada.<br>Poster presentation.                       |

- 06/ 2013**      12<sup>th</sup> Congress of the International Society for Immunology Reproduction (ISIR) hosted by the American Society for Reproductive Immunology (ASRI), Boston, Massachusetts, USA.  
Poster presentation.
- 06/ 2012**      Joint International Congress of the American Society for Reproductive Immunology (ASRI) and the European Society for Reproductive Immunology (ESRI), Hamburg, Germany.  
Poster presentation.
- 06/ 2012**      International training in reproductive sciences and technologies, 3<sup>rd</sup> Jena InTreST-DGRM, Jena, Germany.
- 10/ 2011**      Reproductive biology and Immunology, autumn school, October 2011, Magdeburg, Germany.

## PUBLICATION

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- Chaiwangyen W, Ospina-Prieto S, Photini **Mary S**, Schleussner E, Markert UR, Morales Prieto DM. Dissimilar microRNA-21 functions and targets in trophoblastic cell lines of different origin. *The International Journal of Biochemistry & Cell Biology* (under revision).
- Stanimir Kyurkchiev, Fulvio Gandolfi, Soren Hayrabedyan, Tiziana A. L. Brevini, Roumen Dimitrov, Justine S. Fitzgerald, Asma Jabeen, Milena Mourdjeva, Photini **Mary S**, Patrick Spencer, Nelson Fernandez, Udo R. Markert Stem Cells in the Reproductive System *American Journal of Reproductive Immunology* 67 (2012) 445-462 (Review article).

## CONTRIBUTIONS

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- (2015) **Mary** Stella, Wittaya Chaiwangyen, Justine S. Fitzgerald, Ekkehard Schleussner, Udo R. Markert .The role of PIM1 in trophoblast cells. ASRI. Ontario, Canada.

- (2015) **Mary** Stella, Wittaya Chaiwangyen, JS Fitzgerald, Schleussner E, UR Markert, HTR8/SVNEO spheroids stemness is altered by OSM. International congress on Embryo Implantation and Pregnancy. P34.
- (2014) **Mary** Stella, Justine S. Fitzgerald, Ekkehard Schleussner, Udo R. Markert, Identification of PIM1 in Trophoblastic cells. PLACENTA Volume: 35 Issues: 9 Pages: A105-A105 Meeting Abstract: P2.143-N Published: SEP 2014.
- (2014) **Mary** Stella, Justine S. Fitzgerald, Ekkehard Schleussner, Udo R. Markert, OSM effect on HTR8/Svneo spheroids. PLACENTA Volume: 35 Issues: 9 Pages: A94-A95 Meeting Abstract: P2.108-N Published: SEP 2014.
- (2014) **Mary** Stella, JS Fitzgerald, Schleussner E, UR Markert, HTR8/SVNEO spheroids stemness is altered by LIF. ESRI, Budapest, Hungary.
- (2013) **Mary** Stella, JS Fitzgerald, UR Markert, Identification of PIM kinases upon LIF stimulation in transformed trophoblast cells and choriocarcinoma cells, American journal of Reproductive immunology volume 69, Issue s2, April 2013, Pages 157 P-178, poster presentation.
- (2013) **Mary**. Photini\*, Stella Wittaya Chaiwangyen, Udo R. Markert The possible role of PIM kinases upon LIF stimulation in transformed trophoblast cells and choriocarcinoma cells” Placenta, Volume 34, Issue 2, February 2013, Pages A1-A94 Poster Presentation.
- (2012) **Mary** Stella, Wittaya Chaiwangyen, Sebastian Hoelters, Justine S Fitzgerald, Udo R Markert The possible role of PIM kinases (Pim1, Pim2 and Pim3) upon LIF stimulation in transformed trophoblast cells and choriocarcinoma cells Placenta, Volume 33, Issue 9, September 2012, Pages A1-A137 Poster Presentation.

- (2012) **Mary** Photini Stella, Göhner C, Chaiwangyen W, Pastuschek J, Hoelters S, Fitzgerald J S, Markert UR. Characterization of sphere formation in trophoblast cell (HTR8/SV-neo) to explain their stem cell property. Journal of Reproductive Immunology, Volume 94, Issue 1, May 2012, Page 73. Poster Presentation.
- (2010) **Mary** Stella, Shiva Sivasubramaniam. “Invasive potentials of transformed placental cells and gestational trophoblastic tumor cells –A Comparative analysis of placental versus tumor invasion”. Division of Molecular Biosciences, School of Biomedical and Natural Sciences, Nottingham Trent University, Clifton Lane, Nottingham NG11 8NS, UK.
- (2008) **Mary** Stella, Diagnosis for life style disease –Diabetes Type-II. Loyola College Contributed an article to the Regional Symposium on newer challenges in laboratory.
- (2008) **Mary** Stella, Fungal keratitis-Epidemiology, risk factor & diagnosis, University of Madras paper presentation: Souvenir Silver Jubilee celebration organized by Department of Microbiology, University Of Madras.

## LANGUAGES

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ENGLISH: Fluent  
 HINDI : Fluent  
 TAMIL : First Language  
 GERMAN: B-level

# EHRENWÖRTLICHE ERKLÄRUNG

Hiermit erkläre ich, dass mir die Promotionsordnung der Medizinischen Fakultät der Friedrich-Schiller-Universität bekannt ist,

ich die Dissertation selbst angefertigt habe und alle von mir benutzten Hilfsmittel, persönlichen Mitteilungen und Quellen in meiner Arbeit angegeben sind,

mich folgende Personen bei der Auswahl und Auswertung des Materials sowie bei der Herstellung des Manuskripts unterstützt haben: Prof. Dr. med. Udo Markert und Dr. Wittaya Chaiwangyen, Dr. Diana Morales Prieto.

die Hilfe eines Promotionsberaters nicht in Anspruch genommen wurde und dass Dritte weder unmittelbar noch mittelbar geldwerte Leistungen von mir für Arbeiten erhalten haben, die im Zusammenhang mit dem Inhalt der vorgelegten Dissertation stehen,

dass ich die Dissertation noch nicht als Prüfungsarbeit für eine staatliche oder andere wissenschaftliche Prüfung eingereicht habe und

dass ich die gleiche, eine in wesentlichen Teilen ähnliche oder eine andere Abhandlung nicht bei einer anderen Hochschule als Dissertation eingereicht habe.

Jena 03.07.2015

Stella Mary